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Equine laminitis pain and modulatory mechanisms at a potential analgesic target, the TRPM8 ion channel

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DECLARATION

I hereby declare that the composition of this thesis and the work presented are my own, with the exception of the ATF-3 and NPY immunohistochemistry which was carried out by Emma Jones. The contribution of others is also appropriately credited. Some of the data included in this thesis have been published and are included in the Appendix.

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ABSTRACT

Chronic neuropathic pain, resulting from dysfunction of the nervous system, is a clinical concern in both humans and animal patients. Neuropathic pain is characterised by spontaneous pain, hypersensitivity, manifested as hyperalgesia and allodynia, and refractoriness to conventional analgesics such as non-steroidal anti-inflammatory drugs, thus representing an unmet therapeutic need.

Equine laminitis is a disease that involves the disruption of the dermo-epidermal junction within the hoof, leading to severe pain and lameness, with poor responsiveness to anti-inflammatory therapy. We developed a Quantitative Sensory Testing method, using a novel hydraulically-powered feedback-controlled hoof tester, in order to provide an objective tool for the assessment of mechanical hyperalgesia in laminitic horses. Hoof Compression Thresholds of laminitic horses were significantly lower than those of normal horses and variance component analysis of the data confirmed the reliability of the method. In order to investigate mechanisms underlying laminitis pain, we performed histological studies of peripheral nerves innervating the hoof. Electron micrographic analysis of the digital nerve of laminitic horses revealed a significant reduction in the number of unmyelinated and myelinated fibres together with abnormal morphology. Additionally, cell bodies of sensory neurons innervating the hoof in cervical C8 dorsal root ganglia showed an upregulated expression of the nerve injury marker activating transcription factor-3 (ATF3), neuropeptide Y (NPY), and the TRPM8 channel; each of which has been associated with laboratory models of neuropathic pain.

Previous work has shown that, in a rodent model of neuropathic pain, the TRPM8 channel is upregulated in sensory neurons and its activation by cool temperature, menthol or icilin leads to reversal of the hypersensitive pain state. Further investigation of TRPM8-channel mediated analgesia was aimed at uncovering the molecular mechanisms involved in the activation of this system in sensitised states. It was hypothesised that serotonin, released following inflammation and nerve damage, can enhance TRPM8 channel activity through peripheral 5-HT_{1B} receptors. Calcium fluorometry carried out in HEK293 cells

transfected with the TRPM8 channel and the 5-HT_{1B} receptor revealed that co-administration of a 5-HT_{1B} receptor agonist facilitated the activation of the TRPM8 channel by icilin. Moreover, it appears that this effect is mediated through phospholipase D1 (PLD1), possibly leading to increased production of phosphatidylinositol (4,5-) bisphosphate (PIP_2), a known positive modulator of TRPM8 channel activity. In vitro co-immunoprecipitation studies suggested that the TRPM8 channel, the 5-HT_{1B} receptor and PLD1 physically interact with each other, further providing a molecular basis for their functional co-operation. Calcium imaging carried out in cultured rat DRG cells showed that the 5-HT_{1B} receptor-mediated enhancement of icilin responses at the TRPM8 channel also occurs in sensory cells and is reversed by inhibition of PLD1. Moreover, TRPM8 and the 5-HT_{1B} receptor appear to be physically associated in vivo as shown by their co-immunoprecipitation from spinal cord homogenates. Assessment of nociceptive behavioural reflexes following intrathecal injection of selective pharmacological agents provided further support for the idea of 5-HT_{1B} receptor facilitation of TRPM8 channel responses in vivo. In addition to providing novel evidence of a neuropathic component to equine laminitis and validation of a novel QST method for pain assessment in horses, this study reveals for the first time a physical and functional interaction between the 5-HT_{1B} receptor and the TRPM8 channel.

ABBREVIATIONS

5-HT	serotonin
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
ASIC	acid-sensitive ion channel
ATF-3	activating transcription factor 3
ATP	adenosine triphosphate
BDNF	brain-derived neurotrophic factor
Ca^{2+}	calcium ions
cAMP	cyclic adenosine monophosphate
Ca_v	voltage-gated calcium channels
CCI	chronic constriction injury
CGRP	calcitonin-gene related peptide
CGS	clinical grading system
Cl^-	chloride ions
CNS	central nervous system
CP 94253	5-propoxy-3-(1,2,3,6-tetrahydro-4-pyridinyl)-1H pyrrolo[3,2-b]pyridine hydrochloride
CTb	cholera-toxin B subunit
DEG/ENaC	degenerin/epithelial sodium (Na) channel
DRG	dorsal root ganglia
DMEM	Dulbecco's modified Eagle Medium
EM	electron microscopy
GABA	gamma-aminobutyric acid
GDNF	glial-derived neurotrophic factor
GFRα	GDNF family receptor α
HCT	hoof compression threshold
HIV	human immunodeficiency virus
IASP	International Association for the Study of Pain
IB4	isolectin B4
ICC	intra-class correlation coefficient
IEG	immediate early genes

KCC2	potassium-chloride co-transporter
KCNK	two-pore potassium channel
K^+	potassium ions
K_v	voltage-gated potassium channels
LT	low-threshold
LTP	long-term potentiation
MAGUK	membrane-associated guanylate kinase
MAPK	mitogen-activated protein kinase
mEPSC	miniature excitatory postsynaptic current
Mg^{2+}	magnesium ions
mGluR	metabotropic glutamate receptor
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
Na^+	sodium ions
Na_v	voltage-gated sodium channels
NF-200	neurofilament 200 kDa
NGF	nerve growth factor
NK1	neurokinin 1
NKA	neurokinin A
NMDA	N-methyl-D-aspartate
NPY	neuropeptide Y
NR	NMDA receptor subunit
NRM	nucleus raphe magnus
NS	nociceptive-specific
NSAIDs	non-steroidal anti-inflammatory drugs
P2X	purinergic receptor subtype X channel
PAG	periaqueductal grey matter
PBS	phosphate buffer saline
PEL	primary epidermal lamellae
PG	prostaglandin
PHI	peptide histidine-isoleucine
PI 3-K	phosphoinositide 3- kinase

PI 4-K	phosphoinositide 4-kinase
PI 5-K	phosphoinositide 5-kinase
PK	protein kinase
PLC	phospholipase C
PSL	partial sciatic nerve ligation
PWL	paw withdrawal threshold
QST	quantitative sensory testing
RMM	rostral medial medulla
ROC	receiver operating characteristic
SB2242289	hydrochloride (1'-methyl-5-[[2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]carbonyl]-2,3,6,7 tetrahydrospiro[furo[2,3-f]indole-3,4'-piperidine hydrochloride
SEM	standard error of the mean
SG	substantia gelatinosa
SIA	stress-induced analgesia
SNI	spared nerve injury
SNL	spinal nerve ligation
SP	substance P
SPA	stimulation-produced analgesia
STT	spinothalamic tract
TMP	thiamine monophosphatase
TNF-α	tumor necrosis factor alpha
Trk	tyrosine kinase
TRP	transient receptor potential
TTX	tetrodotoxin
VR1	vanilloid receptor subtype 1
WDR	wide dynamic range

Chapter 1: INTRODUCTION

1.1 Pain definition

The ability to respond to aversive environmental stimuli is a defining attribute of animals. The detection of a noxious, i.e. a damaging or potentially damaging stimulus and the generation of a motor response to avoid it is known as nociception. This term was coined by Sherrington (1903) in order to describe the responses observed in decorticate animals, unable to feel pain. However, nociception can (and generally does) result in the experience of pain, which relies upon higher CNS processing. Pain has been defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (I.A.S.P., 1979). A number of limitations have been identified with regard to this definition and its application to non-verbal humans and animals. Anand and Craig (1996) highlighted that the IASP definition relies heavily on language and self-report. They argued that self-report is just one of the "efferent" responses to pain and that the "inability to communicate does not presuppose inability to feel pain". Molony (1997) has provided a working definition of animal pain that focuses on efferent responses other than verbal self-report: "Animal pain is an aversive sensory and emotional experience representing an awareness by the animal of damage or threat to the integrity of its tissues; it changes the animal's physiology and behaviour to reduce or avoid the damage, to reduce the likelihood of recurrence and to promote recovery". Additionally, Molony's definition acknowledges that pain comprises not only a sensory-discriminative component (relating to stimulus location, intensity and type) but also an emotional component, thereby involving some form of awareness by the animal. Pain is thus associated with stress and suffering and has an obvious impact on animal welfare. In addition, sustained pain may have detrimental effects on physical health, for example by causing metabolic changes that retard and impair wound healing or result in immuno-suppression (Hellebrekers, 2000). Hypersensitive and often persistent (chronic) pain states, which develop following nerve damage or inflammation, are a

particularly important veterinary concern as they occur not only in association with disease states such as lameness but also when adequate analgesia for surgery or routine procedures such as castration and tail docking is not provided (Kent et al., 2000; Lomax et al., 2010; Slingsby et al., 2006).

1.2 Types of pain

Pain has clearly evolved to serve a protective function, signalling actual or potential damage to the organism in order to enhance its chances of survival. Nociceptive pain, also known as physiological pain, is pain that is elicited by a noxious stimulus which generates a response (reflex withdrawal, arousal and autonomic responses). This type of pain is transient and serves as a warning system to escape if possible from impending bodily damage (Scholz and Woolf, 2002). Acute pain is elicited by injury of body tissue and activation of nociceptive transducers at the site of tissue damage. Acute pain is seen after trauma, surgery and in the course of some diseases. This type of pain is short lived (from days to weeks), responds to treatment and does not persist beyond healing. Chronic pain, on the other hand, is also triggered by injury or disease but it is maladaptive, i.e. it does not serve a protective role, it lasts for months or longer and it is less responsive to treatment.

1.3 Pain plasticity

There is not a direct relationship between the intensity of noxious stimuli and the perceived intensity of pain. Melzack and Wall in 1965 proposed the Gate Control Theory of pain which acknowledged the complex relationship between external stimuli and the perceived sensation they evoke. This theory suggested that the central nervous system (CNS) modulates pain signalling, as the spinal cord and the brain act as filtering and processing sites, rather than as passive transmitters and perceivers (Melzack et al., 2001; Melzack and Wall, 1965). Thus, the role of the CNS has been proved crucial for the establishment and maintenance of sensitised pain states (discussed in later sections), and also, in

phenomena that involve pain suppression, such as the so-called stress-induced analgesia (Butler and Finn, 2009).

Studies on the cellular and molecular neurobiology of sensory afferents have also described plasticity of the peripheral nervous system, revealing a wide range of functional phenotypes that are elicited by different tissue conditions, suggesting a complexity that rivals that of the dorsal horn of the spinal cord for processing afferent information (Price and Geranton, 2009; Reichling and Levine, 2009).

Pathophysiological (chronic) pain states that arise following injury and disease are characterised by abnormal pain hypersensitivity, typically manifested as hyperalgesia and allodynia. Allodynia is defined as pain resulting from normally innocuous stimuli, e.g. light touch following sunburn, while hyperalgesia is defined as a heighted response to painful stimuli (Basbaum and Jessell, 2000) (Figure 1.1). A frequent complaint from human patients suffering from chronic pain is spontaneous pain, which is stimulus-independent and clinically manifested as sensations of pins and needles, burning, stabbing, shooting, and paroxysmal pain (Attal and Bouhassira, 2004).

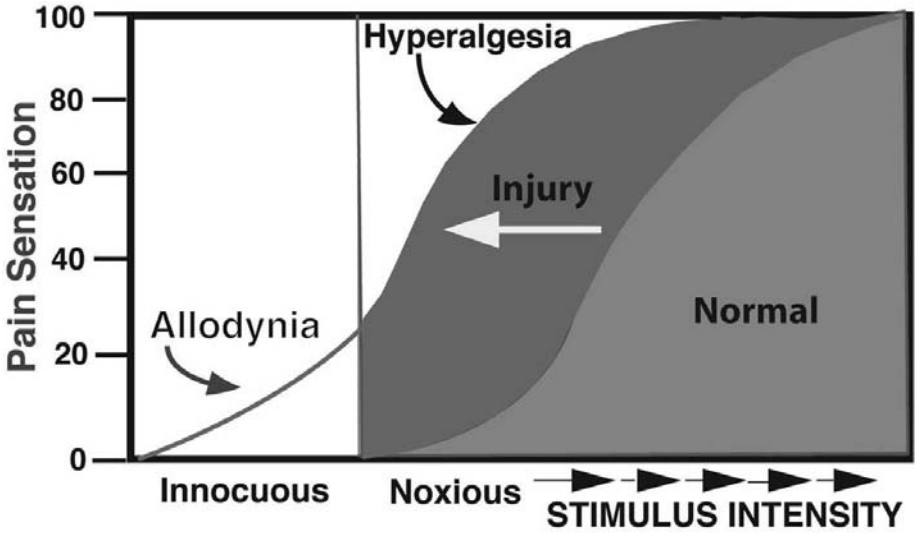

Figure 1.1: *Diagram illustrating the phenomena of allodynia and hyperalgesia. Light-gray area relates to physiological pain whereby the intensity of the pain perceived is proportional to the stimulus intensity. Following injury, this relationship is shifted to the left and thus previously innocuous stimuli now elicit pain (allodynia) and noxious stimuli evoke an increased pain sensation (hyperalgesia). Adapted from Cervero and Laird (1996).*

1.4 Neuropathic pain

Neuropathic pain has been defined by the IASP as a type of chronic pain that is "initiated or caused by a primary lesion or dysfunction in the nervous system" (Merskey and Bogduk, 1994). In categorisation according to the location, neuropathic pain can be central (for example caused by a stroke, spinal cord injury or conditions like multiple sclerosis) or peripheral (as in diabetic neuropathy, post-herpetic neuralgia or phantom-limb pain). In human patients, neuropathic pain is clinically characterised by spontaneous pain (often described as shooting, burning or stabbing) and amplified evoked responses to noxious and non-noxious stimuli (i.e. hyperalgesia and allodynia). Traditional analgesics such as non-steroidal anti-inflammatory drugs (NSAIDs) or opiates are not effective in most neuropathic pain human patients (Namaka et al., 2004). Current treatments for neuropathic pain such as anticonvulsant or tricyclic antidepressants vary in efficacy, may have undesirable side-effects and their mechanisms of action are not completely understood (Baron et al., 2010).

1.5 Laboratory models of neuropathic pain

Current understanding of the mechanisms involved in neuropathic pain has advanced considerably due to the development of laboratory animal models of chronic pain. One of the most frequently used neuropathic pain models is the chronic constriction injury (CCI) model developed by Bennett and Xie (1988). This model involves the loose ligation of the rat sciatic nerve with chromic cat gut ligature which results in the partial occlusion of the epineurial vasculature. Following CCI surgery, rats develop hyperalgesia and allodynia and exhibit nocifensive behaviours indicative of spontaneous pain. Other commonly used models of neuropathic pain are partial sciatic nerve ligation (PSL) which involves tight ligation of about half of the sciatic nerve (Seltzer et al., 1990), spinal nerve ligation (SNL) which involves the tight ligation of the L5 and L6 spinal nerves (Kim and Chung, 1992) and spared nerve injury (SNI) (Decosterd and Woolf, 2000) in which the tibial and the common peroneal nerve, two of the

three distal nerve branches of the sciatic nerve are axotomised, leaving the third branch, the sural nerve, intact.

Additionally, models have been developed to mimic particular disease states that bring about peripheral nerve injury such as, for example, the streptozotocin model of peripheral diabetic neuropathy (Malcangio and Tomlinson, 1998), peripheral demyelination (Wallace et al., 2003), a model of post-herpetic neuralgia induced with varicella-zoster virus (Fleetwood-Walker et al., 1999) or HIV-induced neuropathy (Herzberg and Sagen, 2001).

Laboratory animal models of neuropathic pain have certain limitations. For example, some degree of variation has been described in the nociceptive hypersensitivity exhibited by CCI rats, probably due to the tightness or the type of ligature (Maves et al., 1993; Ro and Jacobs, 1993), making standardisation difficult and limiting comparisons across laboratories. In the PSL model, the magnitude and duration of the pain hypersensitivity varies depending both on the suture material and the strains of rats used (Yoon et al., 1999). Another methodological limitation is that sciatic nerve injury often results in a postural deformity of the ipsilateral paw that is clearly noticeable and renders blind testing impossible. Caudal trunk resection models have been suggested as an alternative to overcome this limitation as testing on the tail can be carried out blindly and consistently (Jaggi et al., 2011).

The validity of animal models of neuropathic pain has also been questioned as they might not mirror some features that are common clinical complaints in human patients suffering from neuropathic pain syndromes (e.g. sleep disturbance, Kontinen et al., 2003). Criticisms have also been focused on the perceived lack of predictive value of animal models of neuropathic pain in drug discovery (Blackburn-Munro, 2004; Rice and Hill, 2006).

Despite their limitations, laboratory animal models of neuropathic pain have greatly contributed to our understanding of the mechanisms involved in neuropathic pain, as described below, and it is considered that they will remain

essential tools in the repertoire of drug discovery programmes (Whiteside et al., 2008).

1.6 Chronic and neuropathic pain in domestic animals

The management of chronic pain in domestic animals remains a largely unmet clinical need in veterinary medicine. The potential for the development of chronic pain as a consequence of disease and surgical procedures raises animal welfare concerns (Lascelles and Main, 2002). Limitations in current strategies to recognise and treat chronic pain contribute to this situation.

Little is known about the incidence of chronic pain in domestic animals. Studies investigating the epidemiology of pain in domestic animals are limited. However, recent studies in dogs and cats have found that, although the most frequently identified type of pain is acute and inflammatory, an incidence of secondary hyperalgesia of 12–18% suggests that central plasticity and sustained pain may be key clinical concerns that should be explored and targeted (Muir, III et al., 2004; Wiese et al., 2005).

Similarly, there is evidence that lame cattle and sheep are in a chronic state of hyperalgesia beyond healing of lesions (Ley et al., 1989; Whay et al., 1998). Beak trimming in poultry has also been associated with long-term physiological and behavioural changes associated with increased pain sensitivity (Breward and Gentle, 1985; Duncan et al., 1989).

In this regard, pre-emptive analgesia may be important in the prevention of chronic pain as it not only aims to reduce nociception but also sensitisation induced by tissue damage. Some evidence for this phenomenon has been successfully observed in animals following administration of opioids, NSAIDs and NMDA receptor antagonists (Lascelles, 2000; Wilson et al., 2005), although its benefits are still debated in human patients (Furlan et al., 2006; Moiniche et al., 2002).

Neuropathic pain is poorly recognized in domestic animals mainly due to difficulties associated with its diagnosis and characterisation (Cashmore et al.,

2009). Suspected neuropathic pain disorders described in domestic animals are associated with sensory abnormalities (especially allodynia and dysaesthesia), abnormal behaviour or locomotion associated with sensory hypersensitivity and unresponsiveness to conventional analgesic therapy, including non-steroidal anti-inflammatory drugs (NSAIDs) and opioids (Cashmore et al., 2009). Examples of disorders reflecting neuropathic pain in domestic animals include orofacial pain syndrome in cats (Rusbridge et al., 2010), syringomyelia in dogs (Rusbridge and Jeffery, 2008) and headshaking in horses (Newton et al., 2000). In many cases, diagnosis has been made from responses to pharmacotherapy that targets neuropathic pain (Davis et al., 2007; Robertson and Lascelles, 2010).

Naturally occurring diseases in domestic animals can be used as models of human pain. Recent reports have suggested that conditions such as osteosarcoma and osteoarthritis in dogs or several musculoskeletal disorders in horses can provide useful comparative data on the aetiopathogenesis of human diseases and can also be used to assess new therapeutic approaches with a potential application in humans (Brown et al., 2005; Innes and Clegg, 2010).

In particular, naturally occurring neuropathic disorders in domestic animals also have the potential to provide clinically relevant models that closely mimic human neuropathic pain conditions. For example, feline diabetes mellitus resembles the disease in humans, both in terms of the observed peripheral nerve pathology and the clinical symptoms, and is considered a useful model for human diabetic neuropathy (Henson and O'Brien, 2006; Mizisin et al., 2007).

1.7 Neural circuits of pain

1.7.1 Primary afferent neurons and nociceptors

Sensory information from the environment is transduced and relayed to the CNS by primary afferent neurons. Primary afferent neurons have their cell body in dorsal root ganglia (DRG) of spinal nerves or in the cranial nerve ganglia in the head. Primary afferent neurons are pseudo-unipolar neurons which have an axonal stalk from which originate both a peripheral axon, whose nerve endings

innervate the skin, muscle, bones and viscera, and a central axon with terminals that synapse with the CNS. This means that peripheral and central terminals are similar biochemically and, thus, primary afferents can send and receive messages from either end, having been described as "a bidirectional signalling machine" (Basbaum et al., 2009). Although this "bidirectionallity" of primary afferents is apparent, for example, through the phenomenon of presynaptic inhibition, it remains to be elucidated whether activation of central terminals can backfire the whole afferent to release neurotransmitters from peripheral terminals.

For the sake of clarity, only cutaneous primary afferents will be discussed here.

According to axon calibre and myelination, parameters that determine conduction velocity, there are two types of cutaneous sensory afferents: large diameter, myelinated A-fibres, which are fast-conducting; and small diameter, unmyelinated C-fibres, which are slow-conducting.

Aβ fibres are large diameter, fast-conducting (> 10 m/s) myelinated fibres and they have been traditionally associated with the detection and conduction of innocuous stimuli (Lewin and Moshourab, 2004). However, there is substantial evidence that a subset of Aβ fibres are nociceptors, i.e. are specifically activated by noxious (damaging or potentially damaging) stimuli (for a review see Djouhri and Lawson, 2004).

However, most nociceptors are primary afferent neurons which give rise to either thinly-myelinated Aδ fibres or unmyelinated C fibres and terminate in naked, unmyelinated nerve endings in the periphery. Aδ afferent fibres have conduction velocities of 2.2-8 m/s in the rat (Harper and Lawson, 1985) and mediate well localised, "first" or "sharp" pain. Small diameter, unmyelinated C fibres, with conduction velocities of < 1.4 m/s in the rat (Harper and Lawson, 1985) mediate dull, poorly localised, "second" or "slow" pain.

Aδ nociceptors are further divided into two types, according to their electrophysiological responses to noxious mechanical and thermal stimuli. Type I Aδ nociceptors have high temperature thresholds (> 50ºC), whereas type II Aδ nociceptors have a much lower heat threshold but a very high mechanical threshold (Beydoun et al., 1996; Simone and Kajander, 1997). Thus, type I fibres

are likely to convey the first pain provoked by noxious mechanical stimuli, while type II are likely to mediate the first pain response to noxious heat.

Most C fibres are polymodal, responding to noxious mechanical, thermal and chemical stimuli (Bessou and Perl, 1969). A subgroup of C fibres, the so-called "silent" nociceptors, are mechanically insensitive but become gradually sensitive during inflammation, probably due to their higher sensitivity to chemical stimuli than other C fibre mechano-heat receptors (Schmidt et al., 1995).

Not all Aδ and C fibres are nociceptors. Aδ fibres are also involved in the detection of innocuous mechanical and thermal stimuli (Iggo, 1969; Lynn and Carpenter, 1982). Similarly, a subpopulation of C fibres responds to innocuous cooling (Iggo, 1969) and another subset is activated by innocuous stroking of the hairy skin (Vallbo et al., 1999). A group of unmyelinated low threshold mechanoreceptors play a particular role in the mechanical hypersensitivity caused by injury (Seal et al., 2009).

Nociceptors can also be classified in terms of the proteins they contain and release (Figure 1.2). Two different populations of C fibres have been described according to their molecular make-up. Peptidergic neurons release substance P, calcitonin-gene related peptide (CGRP) and express the TrkA and p75 receptors, which detect the neurotrophin, nerve growth factor (NGF) (Snider and McMahon, 1998). Non-peptidergic C fibres, on the other hand, bind the lectin *Bandeiraea simplicifolia* IB4 isolectin, express the enzyme thiamine monophosphatase (TMP) and express the c-Ret neurotrophin receptor and a ligand-binding component, a receptor called GDNF family receptor α **(**GFRα), which is activated by glial-derived neurotrophic factor (GDNF) (Airaksinen and Saarma, 2002; Bennett et al., 1998; Snider and McMahon, 1998). Most of the IB4-positive fibres also express G protein-coupled receptors of the Mrg family (Dong et al., 2001) as well as specific purinergic receptor subtypes, such as the $P2X_3$ receptor (Ramer et al., 2001).

Nociceptors can also be characterised according to their differential expression of particular ion channels that are involved in the transduction of specific noxious stimuli, whether they be chemical (e.g. TRPV1, formerly VR1,

and TRPA1), thermal heat (e.g. TRPV1) or thermal cold (e.g. TRPA1), which will be reviewed in section 1.8.1.

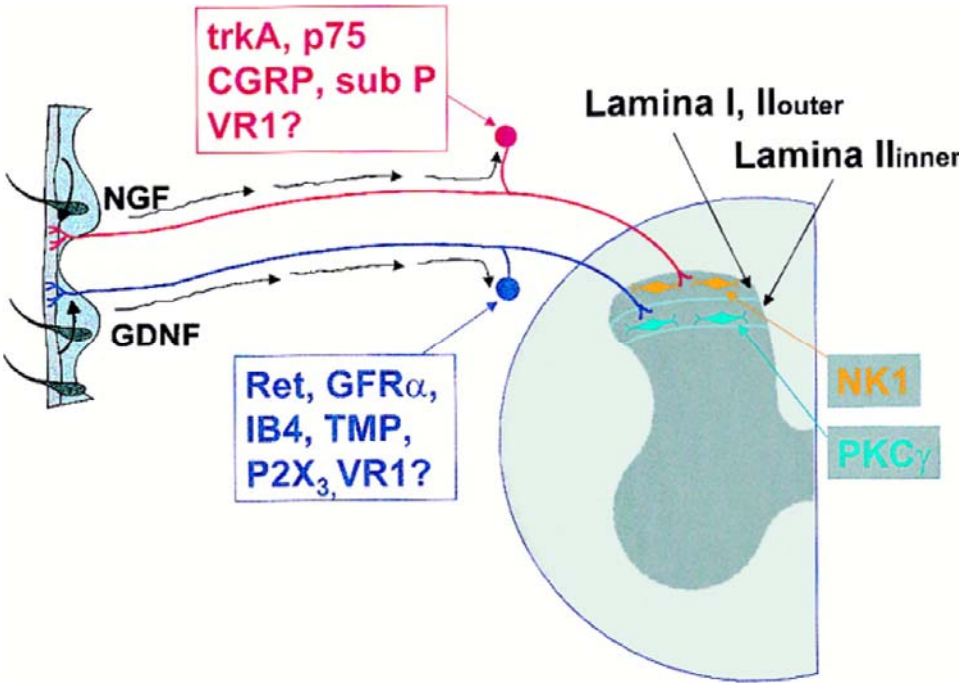

Figure 1.2: *Diagram showing two different populations of cutaneous nociceptors based on their molecular signature. The substance P/CGRP/TrkA population represents roughly 40% of DRG neurons in rodents, whereas the IB4 population represents roughly 30%. Both populations of nociceptors are sensitive to capsaicin, presumably expressing the VR1 (TRPV1) receptor. From Snider and McMahon (1998).*

1.7.2 Nerve injury-related alterations in the properties of primary afferent neurons

A characteristic feature of neuropathic pain disorders is the generation of pain in the absence of an identifiable stimulus that has been linked to the generation of action potentials in primary afferent neurons (Costigan et al., 2009). Indeed, nerve injury can alter the functional properties of primary afferent neurons. Early studies investigating the firing properties of nerves following transection revealed that both neuroma sprouts and DRG neurons can generate spontaneous ectopic discharges (Wall and Devor, 1983; Wall and Gutnick, 1974). Further studies carried out in partial nerve injury animal models of neuropathic pain have shown that abnormal firing occurs both in injured and intact nerve fibres (Ali et

al., 1999; Wu et al., 2002) that might be linked to pain behaviour and changes in gene expression (Obata et al., 2003). Moreover, spontaneous pain can be initiated by ectopic activity in both nociceptors (Bostock et al., 2005) and in low-threshold myelinated fibres (Campbell et al., 1988).

Another phenomenon observed following nerve injury is that of ephaptic transmission whereby nerve fibres and/or cell bodies start to cross-excite each other (Amir and Devor, 1992; Amir and Devor, 2000).

The molecular basis underlying these changes in the excitatory properties and the activity of primary afferent in neuropathic pain will be discussed in section 1.8.3.

1.7.3 Organization of the dorsal horn of the spinal cord

Central terminals of primary afferent neurons synapse with second order neurons in the dorsal horn of the spinal cord. The dorsal horn has been divided into six layers (laminae I-VI) based on the cytoarchitectural organization of its neurons (Rexed, 1954). Most Aδ and C fibres, conveying noxious and thermal information, terminate primarily in laminae I and II of the superficial dorsal horn, with a small number of Aδ fibres terminating in lamina V. Aβ fibres, which convey innocuous information from the periphery, terminate predominately on dorsal horn neurons in laminae III-VI (Todd, 2002).

Peptidergic and non-peptidergic primary afferents also differ in the regions of the dorsal horn in which they terminate (Figure 1.2). CGRP/TrkA–expressing DRG neurons project mainly to lamina I (where the substance P receptor NK1 is heavily expressed) and outer lamina II (IIo), whereas IB4-binding DRG neurons project mainly to the inner lamina II (IIi), characterised by a subpopulation of neurons that express the γ isoform of protein kinase C (PKC_{γ}) (Figure 1.2) (Snider and McMahon, 1998). Although it is not clear if these different projection patterns represent a corresponding functional dichotomy, it has been suggested that laminae I and lamina IIi might play distinct roles in the generation of the hypersensitivity associated with inflammatory and neuropathic pain, respectively (Malmberg et al., 1997; Mantyh et al., 1997).

Three main classes of dorsal horn neurons have been described according to their excitatory responses (Cervero et al., 1976; Handwerker et al., 1975). Low-threshold (LT) neurons (also called class I neurons) are only activated by innocuous stimuli and are primarily found in laminae I, III and IV, receiving inputs from innocuous mechanosensitive Aβ fibres or innocuous temperature information from Aδ/C fibres (Willis and Coggeshall, 1991). Class II neurons, also called wide-dynamic range neurons (WDR), are activated by innocuous and noxious stimuli (Menetrey et al., 1977) and are found predominantly in lamina V, as well as in IV and VI. Finally, nociceptive-specific neurons (NS, also called class III) respond only to noxious stimuli and are mainly located in laminae I and II.

Most lamina I neurons are projection neurons, and they relay noxious information, mainly contralaterally via the spinothalamic tract and subdivisions such as the spinomesenchephalic tract, to various areas in the brain, including the thalamus, periaqueductal grey matter (PAG), the lateral parabrachial area and several parts of the medulla (Burstein et al., 1990; Cechetto et al., 1985; Giesler, Jr. et al., 1979; Lima and Coimbra, 1988; Menetrey and Basbaum, 1987; Menetrey et al., 1982; Spike et al., 2003; Todd et al., 2000).

Lamina II, originally called the *substantia gelatinosa* (SG) due to its appearance, contains both inhibitory and excitatory interneurons that modulate the activity of neurons in other laminae. Both NS and WDR cells have been identified in II_o, whereas only WDR cells and low-threshold mechanical neurons have been reported in II_i (Dubner and Bennett, 1983).

Lamina III and IV neurons are mainly responsive to low-threshold stimuli but some are also WDR cells. Some neurons from this area send dendrites to the superficial dorsal horn. Sprouting of low threshold primary afferent terminals from lamina III into more superficial areas of the dorsal horn has been suggested as a mechanism for the development of neuropathic pain (Mannion et al., 1998). However, further studies have provided a legitimate reason for believing that this

may not be the case because validity of using cholera toxin B subunit (CTb) as a selective tracer for sprouting myelinated fibres following peripheral nerve injury (Shehab et al., 2003). Cells from laminae III-IV from the origins of the spinocervical and postsynaptic dorsal column tracts (Brown, 1983) also contribute to ascending pathways such as the spinothalamic tract (Al-Khater et al., 2008; Willis et al., 1979).

Lamina V has a major role in pain processing since the majority of its neurons are WDR cells that respond to innocuous and noxious input. Moreover, axons from cells in this lamina contribute to ascending pathways, such as the spinothalamic and spinomesencephalic tracts (Dubner and Bennett, 1983).

1.7.4 Effect of nerve injury on the behaviour of spinal cord neurons

Sustained nociceptor activation caused by peripheral nerve injury leads to central sensitisation, a phenomenon which results in enhanced responsiveness of neurons in the spinal dorsal horn (Chapman et al., 1998; Laird and Bennett, 1993; Takaishi et al., 1996; Woolf, 1983). Central sensitisation typically involves an increase in ongoing activity in dorsal horn neurons, an increased excitability resulting in enhanced responsiveness to peripheral stimuli and the enlargement of receptive fields size beyond the injured area, changes which are clinically manifested as allodynia and secondary hyperalgesia (Ji et al., 2003; Suzuki et al., 2000). Central sensitisation is dependent on post-synaptic changes involving N-methyl-D-aspartate (NMDA) receptors, which are similar to those underlying long-term potentiation (LTP), a mechanism involved in memory formation in the hippocampus (Ji et al., 2003). The molecular basis of central sensitisation will be further discussed in section 1.9.3.

Some authors have differentiated between central sensitisation and centralisation of pain, whereby pain is generated by the CNS independently of ongoing peripheral activity (Devor, 2006). Central changes leading to centralisation of pain may include increased excitability, structural changes in synaptic connectivity, loss of inhibitory interneurons and alteration in descending modulatory systems (Costigan et al., 2009).

1.7.5 Ascending pathways

Projection neurons from the dorsal horn of the spinal cord transmit somatosensory information to supraspinal structures through a number of pathways. The spinothalamic tract (STT) is a major ascending pathway for nociceptive and temperature information. STT neurons originate mainly from lamina I and laminae IV-VI and in their majority project to the contralateral ventrobasal and posterior thalamus, where they synapse with cells that project to the somatosensory cortex, which is involved in the sensory discriminative component of pain (Treede et al., 1999; Willis et al., 1979).

Another relevant ascending pathway is the spinoparabrachial pathway, which is mainly comprised of noci-specific neurons with very few neurons responding exclusively to innocuous stimuli (Bester et al., 2000). This pathway mainly originates from cells in lamina I that express the SP receptor NK1 (Bester et al., 2000; Todd, 2002) and are thought to encode the intensity of noxious stimuli (Doyle and Hunt, 1999). The parabrachial area is involved in autonomic regulatory functions and projects to the central nucleus of the amygdala and the hypothalamus, and is thus thought to be involved in autonomic and affective reactions to painful stimuli (Bester et al., 2000).

Other pathways that relay nociceptive information to the brain include the ventral spinothalamic tract, the spinomesencephalic tract and the spinocervical tract. These will be not discussed further.

1.7.6 Descending modulation of pain

The transmission of nociceptive inputs is modulated at all levels of the neuroaxis. Descending modulation of pain was proposed by Sherrington (1906), who believed that the transmission of sensory information critically involved the interaction of inhibitory and excitatory systems. Hagbarth and Kerr (1954) provided the first evidence of modulation of spinal afferent conduction following repetitive stimulation of various areas of the brain. The influence of descending modulation on the processing of nociceptive inputs in the dorsal horn was a prominent feature of the Gate Control Theory, proposed by Melzack and Wall (1965), and was further supported by reports that showed induction of analgesia

through electrical stimulation of the periacqueductal gray (PAG) in the mesencephalon, a phenomenon called stimulation-produced analgesia (SPA) (Akil and Liebeskind, 1975; Reynolds, 1969). Further elucidation of the anatomy and neurochemistry underlying this phenomenon revealed the involvement of endogenous opioids and a serotonergic system originating at the nucleus raphe magnus (NRM), a medullary structure innervated by the PAG that sends projections to the spinal and medullary dorsal horn (Basbaum et al., 1976; Mayer and Price, 1976).

The existence of descending modulatory systems devoted to modifying nociceptive transmission suggests an adaptive mechanism whereby responses to pain are modulated according to their relevance for increasing the organism's chances of survival. Although pain is vital as a warning mechanism for life preservation, there are certain circumstances, for example in situations of extreme danger, in which the inhibition of pain might be more adaptive than its perception. A key phenomenon in this regard is what has been termed stress-induced analgesia (SIA), a suppression of pain sensation in the presence of stressful stimuli (reviewed by Butler and Finn, 2009). The evolutionary advantage conferred by stress-induced analgesia has been suggested, for example, in studies showing that SIA is induced in rats, mice and voles in response to the presence of predators (Kavaliers and Choleris, 1997; Saksida et al., 1993; Sudhakar and Venkatesh, 2001).

Descending modulation of spinal nociceptive transmission can also be facilitatory. A key area involved in descending facilitation is the rostral medial medulla (RMM) and this pathway appears to course through an anatomical pathway independent from the dorsolateral funiculus, which is involved in descending inhibitory pathways (Zhuo and Gebhart, 2002). The RMM has been suggested to play a prominent role in the development of secondary hyperalgesia in a variety of chronic pain states (Urban and Gebhart, 1999). Additionally, selective ablation of RMM cells expressing the μ-opioid receptor prevented the development or reversed the establishment of allodynia in a rat model of neuropathic pain (Porreca et al., 2001). Descending facilitation is initiated by

injury to primary afferents and tissue inflammation (Gardell et al., 2003; Wei et al., 1999) and projection neurons from lamina I have been shown to regulate a descending serotoninergic facilitatory pathway that is mediated by 5-HT_3 receptors in superficial dorsal horn (Suzuki et al., 2002).

1.8 Molecular mechanisms of nociception

Nociceptors form late during neurogenesis and develop from neural crest stem cells, migrating from the dorsal part of the neural tube (Woolf and Ma, 2007). All embryonic nociceptors are NGF dependent and express the TrkA receptor (Marmigere and Ernfors, 2007). Mutations in the TrkA receptor, for example as in the hereditary sensory and autonomic neuropathy type IV, might result in loss of nociceptor neurons and congenital pain hyposensitivity (Verpoorten et al., 2006). Nociceptor differentiation occurs through complex temporal patterns of protein expression. During the perinatal and postnatal period TrkA is downregulated in half of the developing nociceptors which instead start expressing the GDNF receptor Ret and become the nonpeptidergic population of nociceptors (Molliver et al., 1997).

Sensory transduction is carried out by specific molecules located in the membrane of the peripheral terminals of primary afferent neurons. A particularly relevant group of channels in this regard is the transient receptor potential family (TRP), a diverse family of non-selective cation channels. TRP channels were discovered in *Drosophila melanogaster* for their role in phototransduction (Montell and Rubin, 1989). TRP channels are ubiquitously expressed across all kingdoms of life, having been identified in species ranging from yeast to mammals (Ramsey et al., 2006). TRP ion channels appear to subserve a main function as cell sensors, with prominent roles in vision, olfaction, taste, mechanosensation, osmoregulation and thermosensation (Clapham, 2003). Mammalian TRP channels are classified into subfamilies based on sequence homology: TRPC (canonical); TRPV (vanilloid, so named because the first identified member is activated by the vanilloid, capsaicin, the pungent ingredient

of chilli peppers); TRPM (melastatin, named for the role of the first member of this family in melanoma metastasis); TRPA (ankyrin, named for the ankyrin repeats in the N-terminal tail of this subfamily member); TRPP (polycystin, named for its involvement in polycystic kidney disease); and TRPML (mucolipin, so called due to mutations in this channel are associated with mucolipidosis) (Clapham, 2003; Moran et al., 2004; Ramsey et al., 2006). The genetic sequences and structures of TRP are very diverse although they all share a structure based on six transmembrane (TM) domains, with cytoplasmic amino and carboxy termini. TRP channels appear to function as tetramers, and mostly as homomers (Clapham, 2003; Ramsey et al., 2006).

The emergence of transient receptor potential (TRP) channels during development occurs in a time-dependent manner, with TRPV1, TRPV2, TRPM8 and TRPA1 expression first becoming detectable in embryonic mice at E12.5, E10.5, E16.5 and P0, respectively (Hjerling-Leffler et al., 2007; Shibasaki et al., 2010). Although the role of TRP channels during development has not been elucidated it appears that it might go beyond their sensory function. A recent study has suggested that TRPV2 is involved in the regulation of axon outgrowth in both sensory and motor neurons (Shibasaki et al., 2010).

1.8.1 Molecular transduction of noxious stimuli

The molecular basis of noxious stimulus transduction, with a particular emphasis on TRP channels, is briefly reviewed below for each stimulus modality.

Noxious heat transduction

Temperatures over 43ºC are typically perceived as painful, in agreement with firing thresholds of C and type II Aδ fibres. Cloning and functional characterization of the capsaicin (vanilloid) receptor (TRPV1) has provided a molecular basis for noxious heat detection (Caterina et al., 1997). This receptor is highly expressed in sensory neurons, preferentially in Aδ and C fibres that typically express molecular markers of nociceptors (Caterina et al., 1997; Tominaga et al., 1998). Indeed, TRPV1 is activated not only by capsaicin but also noxious heat and protons (low pH is a feature of inflammation) (Caterina

and Julius, 2001). TRPV1 has also been shown to be activated by other chemical agonists, including endogenous lipids, such as the endocannabinoid anandamide, N-arachidonoyl-dopamine, and various metabolic products of lipoxygenases, spider toxins and artificial sweeteners (Riera et al., 2007; Siemens et al., 2006; Suh and Oh, 2005). Thus, TRPV1 acts as a polymodal transducer that integrates a variety of stimuli. The integrative role of TRPV1 is most apparent in hypersensitive pain states associated with tissue damage which will be further discussed in section 1.8.3.

TRPV1 has been considered a promising target for pain relief. For example, TRPV1-containing neurons can be rendered insensitive to further noxious stimulation through desensitisation following the prolonged application of agonist drugs, such as capsaicin (Dray et al., 1990).

The molecular domain conferring thermal sensing to TRPV1 appears to be localised to the C-terminal domain. Indeed, interchange of the C-terminus of TRPV1 and that of TRPM8 (which detects cool temperatures, see below) results in a shift in temperature activation to that typical of the donor, i.e. TRPV1 with a TRPM8 C-terminus activates at low temperatures (around 25°C) and TRPM8 with a TRPV1 C-terminus activates at temperatures around 45°C (Brauchi et al., 2006).

Knock-out studies haves shown that acute noxious thermal detection is not mediated solely by TRPV1 (Davis et al., 2000; Woodbury et al., 2004). Other TRP receptors have been suggested to play a role in heat sensation. TRPV2 shows a temperature activation threshold of around 52ºC and is expressed in a subpopulation of Aδ fibres that respond to high-threshold noxious heat (Leffler et al., 2007; Lewinter et al., 2004; Rau et al., 2007). TRPV3 and TRPV4 are activated at temperatures between 25ºC and 35ºC and mice lacking these receptors displayed altered thermal preference on a platform with graded temperatures, suggesting a role in temperature detection *in vivo* (Chung et al., 2005; Guler et al., 2002).

The identification of molecular transducers of cold temperature in sensory neurons has been aided by research on receptors for natural cooling compounds, such as menthol. Cloning and characterization of the transient receptor potential TRPM8 has shown that this receptor is activated by cooling chemical compounds and also innocuous cold temperatures below 25ºC (McKemy et al., 2002; Peier et al., 2002). A role has also been suggested for TRPM8 as a noxious cold detector, and although the mechanisms underlying these opposing functions are unclear, it has been hypothesised that TRPM8 could be expressed in two distinct populations of sensory afferents, one conveying nociceptive inputs and the other one innocuous cold information (Fleetwood-Walker et al., 2007; Knowlton et al., 2010; Takashima et al., 2010). Although TRPM8 is preferentially expressed in small afferents that are TRPV1-negative but TrkA-positive (Kobayashi et al., 2005; Peier et al., 2002; Story et al., 2003), some studies have reported levels of co-expression of TRPM8 and TRPV1 ranging from 10% to 29% (Obata et al., 2005; Okazawa et al., 2004). Additionally, there are several reports of a subset of trigeminal and DRG cells that respond to both menthol and capsaicin and show some degree of histochemical co-localisation of TRPM8 and TRPV1 (McKemy et al., 2002; Okazawa et al., 2004; Reid et al., 2002; Viana et al., 2002). Since TRPV1 is typically expressed in nociceptors, it could be inferred that activation of TRPM8 in the same cells would be perceived as noxious, thus contributing to cold pain. However, the variable times that cells were maintained in vitro in these studies (during which phenotypic changes may occur), mean they may not necessarily provide an accurate reflection of *in vivo* function.

Further insights into the physiological roles of TRPM8 in cool sensation and pain processing have been gained from TRPM8 knockout studies, which have confirmed a major role of TRPM8 in cool sensation. Experiments carried out using *in vitro* skin-nerve preparations showed that subpopulations of C and Aδ fibres were activated by cooling from 32ºC to 28ºC in wild type mice, but not in TRPM8 knockouts (Bautista et al., 2007). Similarly, sensory ganglion cells from knockout mice showed significantly reduced elevation of intracellular Ca^{2+} in

response to cooling (range 20ºC–10ºC) as well as to application of menthol and icilin (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007).

With regard to their behaviour, TRPM8 knockout mice do not exhibit the preference for temperatures around 30ºC (over those in the range downwards to 15ºC) that is observed in wild type mice (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007), suggesting that TRPM8 is implicated in this avoidance behaviour. Similarly, the licking and/or flinching responses to skin cooling by acetone were consistently reduced in TRPM8-knockout mice (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007). However, it was noted that knockout mice avoided temperatures below 10ºC, suggesting that other receptors must, of necessity, be involved in noxious cold transduction (Daniels and McKemy, 2007).

It seems clear that the majority of TRPM8-expressing cells are not classical nociceptors and might be able to exert a quite different functional influence on pain processing (Fleetwood-Walker et al., 2007). Indeed, TRPM8 has been implicated in an endogenous analgesic system apparent in hypersensitive pain states (Proudfoot et al., 2006). Cool temperatures and chemical cooling-compounds such as menthol have been traditionally used to treat pain. The mechanisms of cooling analgesia will be further discussed in section 1.10.

Another TRP channel, TRPA1 has been shown to be activated by cold temperatures, with an average threshold between 12-24°C in heterologous expression systems, with a maximal activation at temperatures below 20°C (Babes et al., 2004; Story et al., 2003). TRPA1 appears to be a major contributor of cold sensing in vagal afferent fibres and trigeminal ganglion neurons (Fajardo et al., 2008; Karashima et al., 2009).

However, knockout studies have provided opposing results and its role in noxious cold-transduction is still being debated (Bautista et al., 2006; Karashima et al., 2009; Kwan et al., 2006).

Importantly, it has been suggested that discrepancies shown between knockout studies could reflect the diversity of behavioural tests used by different

groups, the use of only male or both male and female mice and the strategies used to generate the knockout animals (Story and Gereau, 2006). Furthermore, knockout animals might exhibit functional compensation by related channels and receptors, so they might not necessarily be useful predictors of a gene's role in disease states (Patapoutian et al., 2009).

A recent TRPM8/TRPA1 double knockout has provided further evidence against TRPA1 mediating noxious cold (Knowlton et al., 2010). TRPM8, however, was confirmed as a key receptor in thermal preference for temperatures ranging between 30ºC and 15ºC (Knowlton et al., 2010). The evidence regarding the role of TRPM8 in noxious cold transduction is less conclusive. Even though knockout mice appeared to have less aversive responses to 5ºC than wild type mice, they could clearly discriminate between this noxious temperature and 30ºC, choosing to spend over 80% of the time on the 30ºC plate (Knowlton et al., 2010).

It is likely that other channels contributing to noxious cold detection are present in the peripheral nervous system. For example, Kv1 potassium channels have been shown to be involved in cold detection in TRMP8-expressing neurons. The proposed model argues for two distinct populations of cold thermosensory neurons based on the levels of expression of TRPM8 and Kv1 and their antagonistic effects on neuronal excitability: one population could express high TRPM8 levels, low or absent Kv1 expression and would be involved in innocuous cold sensing and the other population would be involved in noxious cold detection, expressing low TRPM8 levels and high expression of Kv1 (Madrid et al., 2009).

Voltage-gated sodium channel Nav1.8 has also been implicated in the perception of cold pain (Zimmermann et al., 2007) and a recent knock-out study has suggested a role for Nav1.8 and Nav1.9 in the development of cold allodynia in neuropathic pain models (Leo et al., 2010).

Other candidates come from data from double knock-out mice, lacking TREK-1 and TRAAK, which suggests a role in noxious cold detection for these channels, although their mode of operation has not been fully elucidated (Noel et al., 2009; Thut et al., 2003).

Chemical transduction

Nociceptors respond to noxious chemical substances that are either encountered as irritants in the environment or are released endogenously, for example following tissue damage.

TRP channels have a prominent role in sensory chemo-detection and are activated by plant-derived substances such as capsaicin (TRPV1), menthol (TRPM8) and allyl isothiocyanate (mustard oil) and cinnamaldehyde (present in cinnamon oil) (TRPA1). Mice lacking TRPA1 show greatly reduced sensitivity to "tear gases" and isocyanates that produce pain and inflammation, suggesting a role for TRPA1 as a noxious chemo-transducer *in vivo* (Bessac et al., 2009). Moreover, co-localisation of TRPA1 and TRPV1 in a subgroup of DRG and trigeminal nociceptors, also suggests a role for TRPA1 in nociception (Story et al., 2003).

Sensory neuron terminals also respond to proalgesic agents released endogenously in response to tissue damage or physiological stress.

For example, extracellular ATP induces the depolarisation of sensory neurons, and may play a role in nociceptor activation when released from damaged tissue. ATP activates purinergic receptors, including the members of the P2X family of ligand-gated ion channels (Florenzano et al., 2002; Prasad et al., 2001). The P2X3 receptor is uniquely expressed in sensory neurons, particularly in nociceptors (Chen et al., 1995). Indeed, topical or intradermal application of ATP induces pain (Bleehen, 1978; Coutts et al., 1981).

Another chemical mediator, serotonin is released from mast cells, platelets and endothelial cells following tissue damage (Millan, 1999). Peripherally applied serotonin has been shown to induce pain in humans and to evoke nociceptive behaviours in the rat (Jensen et al., 1990; Taiwo and Levine, 1992). However, the role of serotonin and serotonin receptors (5-HT receptors) in pain processing is complex as serotonin may inhibit and/or facilitate nociceptive inputs depending on the type of nociceptive stimuli and, crucially, the type of 5-HT receptors involved, both peripherally and centrally (Millan, 1999). The role of serotonin and 5-HT receptors in pain will be further discussed in chapter 4.

Mechanical transduction

The identification of the molecular transducers that detect tactile stimuli has proven rather difficult. The variety of mechanical stimuli that organisms are able to sense (ranging from vibration or light touch to noxious pressure) and the many subtypes of neurons responding to them have been factors hindering progress in this area of research (Tsunozaki and Bautista, 2009). However, recent studies have provided new insights into mammalian sensory mechano-transduction, suggesting a possible role for three classes of ion channels.

Studies carried out in the nematode *C.elegans*, have identified protein members of the degenerin/epithelial Na channel (DEG/ENaC) family, mec-4 and mec-10 and associated proteins mec-2 and mec-6 as a mechano-transducing complex in neurons (Chalfie and Sulston, 1981). Far less conclusive results have been obtained in studies investigating DEG/ENaC channel mammalian orthologs, ASIC 1, 2 and 3, present in sensory neurons, as their role as mechano-transducers has not been confirmed by knock-out studies (Drew et al., 2004; Page et al., 2005; Price et al., 2000; Price et al., 2001; Roza et al., 2004).

TRP channels have also been suggested as candidates for mechanical transducers in sensory neurons (Christensen and Corey, 2007) but whether they act directly as transducers or are involved in the modulation of mechano-transduction is not clear (Tsunozaki and Bautista, 2009). Knock-out studies of TRPA1 have provided conflicting results with some reporting no changes in mechanical thresholds and others reporting deficits in mechano-sensation (Bautista et al., 2006; Kwan et al., 2006; Petrus et al., 2007). These studies also investigated the role of TRPA1 in sensitised states, which will be discussed in section 1.8.3.

Lastly, two-pore potassium (KCNK) channels have also been proposed as possible mechano-transducers in mammals. Indeed, several KCNK subunits are expressed in sensory neurons (Medhurst et al., 2001). KCNK2 (also known as TREK1) is expressed in a subset of C fibre nociceptors and is activated by heat, osmotic stretch, and pressure (Alloui et al., 2006). Paradoxically, KCNK2-deficient mice display enhanced sensitivity to heat and mild mechanical stimuli,

but normal responses to noxious mechanical pressure (Alloui et al., 2006). The authors of that study suggest that KCNK2 could counteract the effects of an unidentified transduction channel located in skin sensory cells. Another candidate, KCNK18 is expressed in a subset of peptidergic C fibres and low threshold (Aβ) mechanoreceptors, but its role in mechano-transduction is not clear and might be limited to regulating action potential duration and excitability (Bautista et al., 2008; Dobler et al., 2007).

Since a clear mechano-transducer molecule in sensory neurons has not been explicitly identified so far, it is likely that multiple functionally overlapping transduction complexes are involved in sensing touch and that this might be clarified by further research on mouse strains in which multiple genes have been knocked out (Chalfie, 2009).

1.8.2 Voltage-gated ion channels in nociceptive conduction

The transduction of noxious stimuli results in the generation of receptor potentials that activate a variety of a voltage-gated ion channels involved in the conduction of the activity giving rise to pain. Voltage-gated sodium and potassium channels are crucial for the generation of action potentials which convey nociceptive inputs to synapses in the dorsal horn, whereas voltage-gated calcium channels are critical to the release of neurotransmitters from nociceptor terminals (Basbaum et al., 2009).

Sodium channels

Voltage-gated sodium channels (Na_v) open in response to a change in membrane potential and are responsible for the rapid depolarisation required for the generation of an action potential. Several voltage-gated sodium channels have been shown to be expressed in nociceptors. These channels are classified according to their sensitivity to the sodium channel blocker tetrodotoxin (TTX), $Na_v1.3$ and $Na_v1.7$ (amongst others) are sensitive to TTX (TTX-S) whilst $Na_v1.8$ and $Na_v1.9$ are resistant (TTX-R) (for a review see Krafte and Bannon, 2008). The role of these channels in neuropathic pain is briefly discussed in section 1.8.3.

Calcium channels

A number of voltage-gated calcium channels (Ca_v) are expressed in nociceptors where they evoke an increase in intracellular calcium in response to depolarization. In nociceptors, Ca_vs are involved in the release of neurotransmitters such as substance P and CGRP (Lee et al., 2005). Calcium channels are heteromeric proteins composed of α1 pore forming subunits and the modulatory subunits α2δ (which regulates the kinetics of activation and inactivation), α2β or α2γ (Basbaum et al., 2009). Calcium channels can be broadly divided into two groups based on their activation thresholds. High threshold voltage-gated calcium channels include the L (Ca_v1), type and the N, R and P/Q (Ca_v2) types; low threshold voltage-gated calcium channels are the T (Ca_v3) type calcium channels (for a review see Gribkoff, 2006). The high threshold N type, $Ca_v2.2$ channel is highly expressed in nociceptors and is considered one of the most important voltage-dependent calcium channels in pain processing (Gribkoff, 2006). Additionally, knockouts of the α1B subunit specific to this channel showed reduced reflex responses to noxious mechanical and thermal stimuli and reduced pain behaviour in the second phase of the formalin response, suggesting a central role where this channel is abundant on presynaptic terminals (Kim et al., 2001).

1.8.3 Peripheral molecular mechanisms of neuropathic pain

Following tissue damage and injury a peripheral hypersensitive state develops which involves reduced thresholds for firing and increased excitability of nociceptors. The molecular mechanisms underlying peripheral hypersensitivity include the sensitisation of nociceptors and activation of silent nociceptors in the presence of inflammatory mediators, altered expression of neuropeptides and their receptors, expression of new receptors and changes in the expression, composition or localisation of voltage-gated ion channels.

Peripheral sensitisation

Peripheral sensitisation is dependent on inflammatory mediators such as prostaglandin E_2 (PGE_2), bradykinin, ATP, protons, nerve growth factor (NGF) and cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-1β that are released from non-neuronal cells (fibroblasts, mast cells, neutrophils, monocytes and platelets) as well as from nerve terminals (Basbaum et al., 2009). If nerve injury is involved, mediators can also be released by Schwann cells and damaged axons (Campana, 2007). These inflammatory mediators are detected by specific receptors in the primary sensory neurons including: G protein-coupled receptors for PGE_2 and bradykinin, ionotropic receptors for ATP and protons, and tyrosine kinase receptors for NGF and cytokines (Ji and Strichartz, 2004; Woolf and Costigan, 1999).

Peripheral sensitisation can result from the direct action of the inflammatory mediators on particular ion channels involved in nociception or by initiating transcriptional changes that result in changes in protein expression. For example, NGF induces hypersensitivity to heat and mechanical stimuli. NGF acts directly on peptidergic C fibre nociceptors, which express the high-affinity NGF receptor tyrosine kinase TrkA and the lower affinity NGF-binding protein, p75. Interaction of NGF with its receptors induces the activation of downstream signalling pathways (including phospholipase C (PLC), mitogen-activated protein kinase (MAPK) and phosphoinositide 3- kinase (PI 3-K) that lead to the functional potentiation of target proteins, for example TRPV1, resulting in an enhancement of heat sensitivity (Chuang et al., 2001 ; Zhang et al., 2005). NGF also induces upregulation of pronociceptive neuropeptides such as substance P and CGRP (Donnerer et al., 1992; Woolf et al., 1994). In addition, transport of the NGF-TrkA complex to the nucleus of the nociceptive neuron results in increased expression of a number of ion channels that contribute to the excitability of nociceptive neurons, including TRPV1 and the $Na_v1.8$ voltage-gated sodium channel subunit (Chao, 2003; Mamet et al., 2002; Ramer et al., 2001).

TRP channels in neuropathic pain

TRP channels have not only been revealed as the major group of sensory neuron transducers but also as crucial elements in the development of hypersensitive pain states following injury. As described above, following injury there are changes in TRPV1 expression and function. The role of TRPV1 as an integrator of inflammatory mediators has been confirmed by the failure of knock-out mice to develop thermal hyperalgesia in inflammatory pain models (Caterina et al., 2000; Davis et al., 2000). Indeed, TRPV1 antagonists have been shown to provide pain relief in models of inflammatory pain (Krause et al., 2005).

TRPV1 antagonists have also been reported to reduce pain sensitivity in models of neuropathic pain (Culshaw et al., 2006). However, there are not conclusive data on the expression levels of TRPV1 following nerve injury, with some studies reporting an increase and others reporting a decrease (Kim et al., 2008; Michael and Priestley, 1999). Interestingly, a recent study has indicated that the regulation of TRPV1 mRNA levels following nerve injury might be dependent on genotype, as increased, decreased or unaltered levels were observed in different mouse strains (Persson et al., 2010). In models of diabetes-induced neuropathic pain, TRPV1 expression is increased during the hyperalgesic phase and mice lacking TRPV1 fail to develop streptozotocin-induced thermal hyperalgesia (Pabbidi et al., 2008). However, TRPV1 did not appear to be required for the development of hypersensitivity in the PSL model of neuropathic pain (Caterina et al., 2000).

It has been suggested that the role of TRPV1 in neuropathic pain may include sensitization of intact peripheral terminals (through the pathways described above), ectopic activity in injured axons (through activation of axonal TRPV1 receptors) or a contribution to the modulation of transmitter release (Patapoutian et al., 2009). Indeed, a recent study has suggested a role for TRPV1 channels in the modulation of synaptic transmission through its activation by endocannabinoids, such as anandamide (Sagar et al., 2010).

TRPA1 also appears to play a role in hypersensitive states associated with chronic pain. Nerve injury, Complete Freund's adjuvant (an inflammatory

model) and NGF increase TRPA1 expression and activity in sensory neurons (Diogenes et al., 2007; Ji et al., 2008; Obata et al., 2005). Moreover, antisense knockdown of TRPA1 results in a reduction of cold hyperalgesia in both inflammatory and neuropathic pain models (Katsura et al., 2006; Obata et al., 2005). It has been suggested that increased sensitivity to cold in the SNL model could be related to an increased expression of TRPA1 in Aδ fibres (Ji et al., 2008; Ji et al., 2007).

TRPM8 expression increases in DRG neurons ipsilateral to injury in the CCI model of neuropathic pain and there is an enhancement in expression in small myelinated fibres (Frederick et al., 2007; Proudfoot et al., 2006). However, no alterations in TRPM8 expression were reported in the SNL model of nerve injury, nor in a model of inflammation (Katsura et al., 2006; Obata et al., 2005). Similarly, there are reports showing either no change or a reduction on the levels of mRNA transcripts for both TRPM8 and TRPA1 in neuropathic pain (Caspani et al., 2009; Persson et al., 2010; Staaf et al., 2009). Although a role has been suggested for TRPM8 as a candidate mediating cold hypersensitivity in neuropathic pain (Colburn et al., 2007; Xing et al., 2007), other reports suggest that neither TRPM8 nor TRPA1 are involved in cold allodynia (Caspani et al., 2009; Namer et al., 2008).

The role of TRPM8 in nociceptor sensitisation has been questioned. Indeed, inflammatory mediators that induce sensitisation of other TRP channels, result in suppression of TRPM8 function. For example, PKC activation and phosphatidylinositol 4,5-bisphosphate depletion (which are signalling events downstream of bradykinin receptors) and protein kinase A (PKA) activation (which occurs downstream of PGE_2 receptors) abolish or reduce TRPM8 activity (De Petrocellis et al., 2007; Liu and Qin, 2005; Rohacs et al., 2005).

Voltage-gated ion channels and further peripheral mediators in neuropathic pain

Following nerve damage there are changes in primary afferent neurons in terms of their neurotransmitter content and altered gene expression that result in alteration in their properties and activity leading to hyperexcitability. Alterations in the level of expression, cellular localization and distribution of sodium

channels are strongly associated with neuropathic pain (Attal and Bouhassira, 2006). Damaged axons may start to generate ongoing ectopic activity due to the accumulation of sodium channels around the site of injury (Devor et al., 1993); however, so far, no specific sodium channel has been positively associated with hyperexcitability in damaged nerves (Nassar et al., 2006). Pharmacological treatment using sodium channel blockers, such as some antiepileptic drugs or local-anaesthetics, has been shown to provide anti-hyperalgesic effects in patients suffering from neuropathic pain (Attal and Bouhassira, 2006).

Voltage-gated calcium channels have also been implicated in neuropathic pain states. $Ca_v2.2$ and α2δ subunits are upregulated in DRG neurons following nerve injury, suggesting a role in the pathological signal transmission that characterises neuropathic pain states (McGivern, 2006).

Another phenomenon observed following nerve injury is that of ephaptic transmission whereby nerve fibres and/or cell bodies start to cross-excite each other (Amir and Devor, 1992; Amir and Devor, 2000). It has been suggested that cross-excitation might be mediated by neuropeptides, particularly neuropeptide Y (NPY) (Hokfelt et al., 2007). Cross excitation between A and C fibres is correlated with increased NPY in large DRG neurons and the increase in NPY receptor Y2 in small cells following nerve injury (Noguchi et al., 1993; Wakisaka et al., 1991; Wakisaka et al., 1992; Zhang et al., 1997).

1.8.4 Role of the sympathetic nervous system in neuropathic pain

In addition to changes within sensory nerves, sympathetic efferents are able to activate sensory afferents when the latter start to express α2-adrenoceptors following nerve injury (Devor et al., 1994; Xie et al., 1995). Noradrenergic axon sprouting into DRG and functional coupling with the somata of primary sensory neurons has been shown to occur following peripheral nerve injury (McLachlan et al., 1993; Shinder et al., 1999). Recent studies using chemical and surgical sympathectomy in the SNI model suggest that sympathetic input is not involved in the establishment of neuropathic pain but is involved in the maintenance of cold allodynia (Pertin et al., 2007; Zhao et al., 2007).

1.9 Spinal molecular mediators of nociception and chronic pain

1.9.1 Glutamate neurotransmission in the dorsal horn

Glutamate is the main excitatory neurotransmitter in the dorsal horn released from the terminals of primary afferents. Spinal cord neurones responsive to glutamate are predominantly located in LI and LII of the dorsal horn (Schneider and Perl, 1988) and their response is mediated by post-synaptic ionotropic glutamate receptors which include NMDA (N-methyl-D-aspartate), AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate) and kainate receptors as well as by metabotropic glutamate receptors (Bleakman et al., 2006). Glutamate is also released by excitatory interneurons (Sorkin and McAdoo, 1993).

AMPA and kainate receptors mediate fast excitatory transmission in the dorsal horn, while the NMDA receptor is functionally blocked by Mg^{2+} in a voltage-dependent manner and this inhibition is removed upon sufficient depolarisation of the postsynaptic membrane (Mayer et al., 1984). As the main excitatory neurotransmitter involved in nociceptive transmission, much research has been focused on the role of glutamate, its receptors and the signalling pathways downstream of receptor activation, together with their contribution to the development and maintenance of chronic pain states.

Metabotropic glutamate receptors (mGluRs) are G protein-coupled receptors, comprising 8 subtypes (mGluR 1-8), which are categorised into 3 groups depending on their subsequent signalling pathways and their structural and pharmacological characteristics (Neugebauer, 2002). Activation of group I (mGluR 1 and 5) receptors leads to the activation of PLC, and subsequent release of Ca^{2+} from intracellular stores, whereas group II (mGluR 2 and 3) and group III (mGluR 4/6-8) are negatively coupled to adenylate cyclase, and thus inhibit cAMP (cyclic adenosine monophosphate) formation and PKA activation (Neugebauer, 2002). Generally, activation of group I mGluRs increases neuronal excitability and can enhance ionotropic glutamate receptor activity (Bleakman et al., 1992) by mechanisms such as receptor phosphorylation, whereas groups II and III are generally inhibitory (Bleakman et al., 2006).

1.9.2 GABA and glycine influences in the dorsal horn

Gamma-aminobutyric acid (GABA) and glycine are neurotransmitters with generally inhibitory actions that are released by interneurons within the spinal cord. GABA acts on ionotropic $GABA_A$ receptors and metabotropic $GABA_B$ receptors located pre-synaptically on primary afferent terminals as well as on spinal neurons especially within the superficial dorsal horn (Todd and Sullivan, 1990; Todd et al., 1996). In normal conditions, GABA and glycine inhibit the transmission of nociceptive signals from primary afferent nociceptors to postsynaptic projection neurons (pre-synaptic inhibition) and reduce the excitability of projection dorsal horn neurons (post-synaptic inhibition), thus acting as modulators or "gate-keepers" of nociceptive transmission. Thus, pharmacological blockade of GABAergic and glycinergic influences results in tactile allodynia and increased synaptic transmission from Aβ fibres to nociceptive lamina I neurons (Baba et al., 2003; Torsney and MacDermott, 2006).

Pre-synaptic inhibition appears to be mediated solely by GABAergic synapses, whereas both GABA and glycine are involved in post-synaptic inhibition (Zeilhofer et al., 2009). $GABA_A$ receptors are ligand-gated chloride channels and their activation increases membrane Cl^- conductance resulting in hyperpolarisation of the cell, whilst $GABA_B$ receptors are linked to Ca^{2+} and K^+ channels. Their activation results in a decrease in Ca^{2+} currents and an increase in K^+ currents that hyperpolarise the cell and reduces transmitter release (Malcangio and Bowery, 1996). Glycine receptors contain a chloride-permeable ion channel and activation of these receptors hyperpolarises neurones (Hamill et al., 1983). But glycine does not only serve an inhibitory role. Glycine is an obligatory co-agonist of typical NR1 and NR2 subunit-containing NMDA receptors and in addition, NMDA receptors containing the NR3 subunit can be activated by glycine, independently of glutamate (Chatterton et al., 2002).

Inhibitory dorsal horn neurons are activated by input from both nociceptive and non-nociceptive primary afferent nerve fibres and through descending antinociceptive pathways (Willis and Coggeshall, 1991). There is also evidence for the existence of direct inhibitory GABAergic and glycinergic pathways

descending from the rostral ventromedial medulla (Antal et al., 1996; Kato et al., 2006).

1.9.3 Central sensitisation in chronic pain states

Sustained nociceptor activation caused by peripheral nerve injury and inflammation leads to central sensitisation, where enhanced responsiveness of neurons in the spinal dorsal horn is thought to underlie chronic hyperalgesia and allodynia (Chapman et al., 1998; Kidd and Urban, 2001; Laird and Bennett, 1993; Takaishi et al., 1996; Woolf, 1983). Long-term hyper-excitability of dorsal horn neurons has been attributed to an increased release of excitatory neurotransmitters and alterations in their receptors and also to a decrease or disruption of afferent and descending inhibitory mechanisms in the spinal cord (Saade and Jabbur, 2008).

NMDA receptors play a major role in the development and maintenance of central sensitisation (Coderre and Melzack, 1992; Leem et al., 1996; Sotgiu and Biella, 2002; Woolf and Thompson, 1991). NMDA receptors are abundantly expressed in the dorsal horn of the spinal cord and NMDA receptor subunits NR1 and NR2B are preferentially concentrated in the superficial dorsal horn. NMDA receptor channels are ordinarily blocked by Mg^{2+} and require depolarisation-induced removal of this block as well as glutamate binding in order to open. Depolarisation by increased input from hyperexcitable afferents following injury removes the Mg^{2+} block resulting in the opening of the channel and the entrance of Na^{+} and Ca^{2+}, which will lead to further depolarisation and the activation of intracellular signalling cascades, causing the development of long-term changes in neuronal plasticity. NMDA receptors also interact with specific synaptic proteins that have scaffolding, cytoskeletal, adapter and signalling functions (Husi et al., 2000).

Of particular interest is the suggestion that distinct NMDA receptor–adaptor proteins of the membrane-associated guanylate kinase (MAGUK) family may play different roles in neuropathic and inflammatory pain (Garry et al., 2004; Garry et al., 2003). Although both AMPA and NMDA receptor antagonists have anti-nociceptive properties, the ubiquity of these receptors across the CNS and the associated psychotropic side effects of NMDA antagonists such as ketamine,

severely hamper their use as analgesics. The development of specific NMDA antagonists targeting specific receptor subunits holds promise but is still far from clinical application (Chizh et al., 2001). Continuing studies on glutamate receptor interacting proteins in the CNS in chronic pain states may provide novel and selective analgesic targets.

1.9.4 Spinal disinhibition in chronic pain states

It has been proposed that peripheral nerve injury may lead to a loss of local inhibitory control (disinhibition). This may be due to the death of inhibitory interneurons and/or decreased expression of both the inhibitory neurotransmitter GABA, and of the GABA and opioid receptors, resulting in increased dorsal horn neuron excitability (Sugimoto et al., 1990; Woolf and Mannion, 1999). However, there is some evidence against the role of inhibitory neurons in neuropathic pain, as neuronal loss has been shown not to be necessary for its development (Polgar et al., 2003).

An alternative mechanism has been suggested based on the involvement of the K^+ Cl^- co-transporter KCC2 that regulates chloride homeostasis. A reduction in expression levels of KCC2 would alter the transmembrane anion gradient, shifting GABA-mediated signalling from hyperpolarising to depolarising. Indeed, downregulation of KCC2 has been reported in dorsal horn neurons ipsilateral to peripheral nerve injury, providing a potential mechanism underlying hyperexcitability of lamina I neurons (Coull et al., 2003). This downregulation appears to be mediated by BDNF released from the microglia (Coull et al., 2003).

The importance of glial cells in chronic neuropathic pain has recently been highlighted (Watkins et al., 2007), with microglial activation observed in the dorsal horn after peripheral nerve injury appearing to contribute to the allodynia associated with chronic neuropathic pain (Tsuda et al., 2003).

Following nerve injury there are also changes in the descending modulatory pathways. For example, dorsal horn neurons are less sensitive to inhibition by μ-opioid agonists (Kohno et al., 2005) and there is a shift of descending serotonergic inputs towards facilitation (Vera-Portocarrero et al., 2006). The role

of serotonin receptors in nociceptive processing will be further discussed in chapter 4.

1.10 Mechanisms of cooling-induced analgesia

The application of cold temperature as analgesic therapy has been historically acknowledged by classical medical literature with reports from, for example, Hippocrates, Galen and Avicenna (cited in Aziz et al., 2000; Meeusen and Lievens, 1986). The application of cold temperatures remains a therapeutic option nowadays, with clinical trials showing cooling to provide relief for chronic back pain, dental pain, post-operative pain, orthopaedic pain and for paediatric pain (Belli et al., 2009; Greenstein, 2007; Henning and Firoz, 2010; Levy and Marmar, 1993; Ross and Soltes, 1995; Sauls, 1999).

Cold therapy is also commonly used in the treatment of inflammation and soft tissue injuries in the horse. More specifically, the application of cold has been recommended in the treatment of acute tendon injuries and suspensory desmitis (Hunt, 2001) and laminitis (van Eps and Pollitt, 2004).

Plant-derived compounds that evoke a cooling sensation, such as menthol, have also been used as analgesic therapies. Topical mint oil is used to relieve neuralgia in both traditional Chinese and European herbal medicine (Patel et al., 2007). Menthol has been reported to provide relief in human patients suffering from migraines (Borhani et al., 2007). Additionally, a role for using menthol in neuropathic treatment has recently emerged. Application of topical peppermint oil (containing 10% menthol) has been reported to be effective in a case of postherpetic neuralgia (Davies et al., 2002). In case reports, this compound has also been shown to be effective for the treatment of chemotherapy-induced neuropathic pain (Colvin et al., 2008) and enabling continuation of carboplatin chemotherapy otherwise limited by its neuropathic side effects (Storey et al., 2010). Finally, menthol has been reported to alleviate cold allodynia in patients with neuropathic pain (Wasner et al., 2008).

A number of menthol-containing liniments are used for treating horses, for example for muscle sprains, but there is a lack of systematic analysis of their effectiveness in the veterinary literature.

1.10.1 Neural circuits mediating cold temperature sensation

Two distinct populations of primary afferent fibres that are responsive to cold temperatures have been described. One population is activated by low-threshold 'cool' temperatures (of around 20ºC–35ºC) (Dubner et al., 1975; Kenshalo and Duclaux, 1977), whereas another population is activated by high-threshold noxious cold temperatures of <15ºC, which are generally perceived as painful (Georgopoulos, 1976; LaMotte, 1988). Similarly, experiments carried out in sensory neurons in culture have identified two distinct populations of cold-sensitive neurons, one responding to low-threshold cool temperatures (with an activation threshold of around 29ºC) and one responding to high-threshold cold temperatures (with an activation threshold of around 20ºC) (Nealen et al., 2003; Reid et al., 2002; Thut et al., 2003).

At the spinal cord level, processing of innocuous and noxious cold temperatures is also differentiated. A subset of neurons in the superficial dorsal horn responds specifically to innocuous cold temperatures and receive input mainly from Aδ fibres (Burton, 1975; Iggo and Ramsey, 1974). Noxious cold, on the other hand, activates a subset of dorsal horn neurons that also respond to heat and noxious pinch (Christensen and Perl, 1970). The ascending axons of cooling-specific cells and those from noxious cold-activated multireceptive cells have different conduction velocities and different thalamic terminations (Craig and Dostrovsky, 2001). Moreover, distinct subpopulations of thalamic neurons have also been identified that respond to cool, but not noxious cold, or alternatively to noxious cold but not to cool temperatures (Bushnell et al., 1993) providing a physiological basis for findings from psychophysical studies in humans and monkeys that show perceptual differentiation between cool and noxious cold stimuli (Chen et al., 1996b; Rainville et al., 1999).

Further evidence of independent processing of innocuous *versus* noxious cold has recently been provided in relation to how these inputs can be differentially modulated by the periaqueductal gray (Leith et al., 2010). In that study,

stimulation of the ventrolateral-PAG inhibited noxious responses to a cold stimulus. Moreover, innocuous cold information transmitted by non-nociceptive and wide-dynamic-range neurons remained unaltered, whereas noxious cold information transmitted by wide-dynamic range and nociceptive-specific neurons was significantly depressed.

1.10.2 TRPM8-mediated cooling-induced analgesia

Cloning and characterisation of the TRPM8 channel as the transducer of cool temperatures and of chemical cooling by natural compounds such as menthol or synthetic agents such as icilin and the menthol analogue, WS-12 (Ma et al., 2008; McKemy et al., 2002; Peier et al., 2002) has provided a molecular target for investigating the mechanisms underlying cooling-induced analgesia. As described before, TRPM8 is expressed by a subset (10%–15%) of small diameter primary afferents in spinal DRG and in trigeminal ganglia and can be activated by cooling or by menthol (Madrid et al., 2006; McKemy et al., 2002; Peier et al., 2002; Proudfoot et al., 2006; Reid et al., 2002). In the spinal cord, TRPM8 activation by menthol or cooling results in an increased frequency but not amplitude of miniature excitatory postsynaptic currents (mEPSCs), suggesting that the channel's location is presynaptic (Baccei et al., 2003; Tsuzuki et al., 2004).

TRPM8 activation, using peripherally (topically) or centrally (intrathecally) applied TRPM8 agonists menthol and icilin in the CCI model of chronic neuropathic pain, results in a reduction of sensitised reflex pain behaviours. Moreover, TRPM8 activation reduces the hyper-responsiveness generally observed in dorsal horn neurons following CCI surgery (Proudfoot et al., 2006). Similar results are observed upon cooling of the skin at the typical range of temperatures for TRPM8 activation (Proudfoot et al., 2006). Importantly, reflex responses were unchanged in both the uninjured contralateral limb of CCI animals and in normal animals without a sensitized pain state.

Confirmation of the role of TRPM8 in cooling-induced analgesia was provided by the failure to observe icilin-induced reversal of sensitisation following antisense knockdown of TRPM8 (Proudfoot et al., 2006).

Icilin is also capable of producing analgesia in other chronic pain models, such as the Complete Freund's Adjuvant inflammatory pain model and a neuropathic pain model of demyelination following the application of lysolecithin (Proudfoot et al., 2006).

Further confirmation of a TRPM8-mediated analgesic effect in inflammatory models has been provided by a TRPM8 knockout study (Dhaka et al., 2007). The reduction in nocifensive behaviours produced by cooling the plantar\surface of the hind-paw (17ºC) in wild-type mice in which the hindpaw had been injected with formalin was absent in formalin-injected TRPM8-nulls (Dhaka et al., 2007).

The effects of TRPM8 activation on central processing of pain have not been fully elucidated. However, it appears that TRPM8-mediated analgesia involves the activation of inhibitory glutamate receptors. Indeed, blockade of these receptors with selective mGluR Group II or III antagonists prevented the icilin-mediated reversal of reflex behaviour sensitization (Proudfoot et al., 2006). This suggests that the central analgesic influence of TRPM8 activation in neuropathic pain is mediated by mGlu Group II and III receptors. Moreover, dorsal horn application of a mGluR Group II and III antagonist prevented the reduction in noxious stimulus-induced firing that was caused by peripheral application of icilin (Proudfoot et al., 2006). Interestingly, application of the opioid receptor antagonist, naloxone, had no effect on icilin-mediated analgesia, suggesting that this phenomenon is independent of the classical opioid analgesic system (Proudfoot et al., 2006).

1.11 Pain assessment in animals

Objective and quantitative evaluation of pain in animals is an essential requirement in a variety of contexts ranging from veterinary clinical assessment to biomedical research. Pain assessments carried out by veterinarians are mostly subjective and therefore dependent on the skills and the expertise of the clinicians. In the past few years, there has been a major drive to develop objective pain assessment methods. They have mainly been based on physiological indices, evoked-responses to standard stimuli through quantitative sensory testing (QST) and behavioural quantification.

1.11.1 Physiological measures of pain

Pain is a stressor and as such it induces a biological stress response, involving the recruitment of the sympatho-adrenal and the hypothalamic-pituitary-adrenal systems. Physiological signs generally associated with pain include pupil dilatation, increased respiratory rate, changes in blood pressure and heart rate. Increases in heart rate can be associated with acute pain in many species (Dobromylskyj et al., 2000; Sanford et al., 1986), however, no differences in heart rate were found after arthroscopic surgery in horses (Raekallio et al., 1997). A reduction in heart rate variability (HRV) has been associated with pain in human infants and adults and it has been suggested that alterations in HRV may allow grading of the level of distress caused by pain (Cain et al., 2007; Lindh et al., 1999). A recent study evaluating the association between HRV and behavioural measures of pain in laminitic horses concluded that assessment of HRV offers reliable complementary evidence for pain assessment, although it may also be affected by other factors, such as movement, eating or mental state (Rietmann et al., 2004). Indeed, the main limitation of using physiological measures to assess pain is that they can be altered by numerous other factors and so they are not specific to pain.

The physiological (endocrine) responses to painful procedures such as dehorning of cattle (McMeekan et al., 1998), castration and tail docking in lambs (Molony and Kent, 1997), have also been investigated. Measurement of stress

hormones such as cortisol has been useful in farm animal species as an indirect indicator of acute pain, although they present some limitations such as their lack of specificity to pain and the occurrence of a ‘‘ceiling-effect’’ (Mellor et al., 2000; Molony and Kent, 1997). Measurement of plasma corticosteroid levels can be useful in conjunction with behavioural quantification when assessing the relative effects of a range of painful treatments that elicit injury-specific behaviours (Lester et al., 1996). Although, as mentioned above, these are indirect markers of pain, corticosteroid levels show graded responses to a range of procedures in lambs, and they correlate with behavioural markers, providing valuable supportive measures for the assessment of the relative pain intensity caused by such procedures (Lester et al., 1996).

1.11.2 Quantitative sensory testing (QST)

Quantitative Sensory Testing (QST) techniques are routinely used to assess particular aspects of sensory function in the evaluation of human neurological function. QST methods are psychophysical, non-invasive tests based on the application of standard physical stimuli and the end-point of the test is determined by the patient’s report of stimulus detection or perception of a change in its intensity.

In animals, QST techniques have been adapted by using evoked nociceptive responses as a means of assessing changes in the sensitivity of tissues to potentially painful stimulation. In this context, QST involves the controlled application of standard noxious stimuli (mechanical or thermal) at particular anatomical locations in order to elicit a withdrawal or avoidance reaction, which determines the endpoint of the test. Common parameters measured in QST are reaction time (time elapsed from the onset of the stimulus to the withdrawal response) and the nociceptive threshold (the minimum magnitude of the stimulus required to elicit an avoidance reaction).

QST techniques are commonly used to detect nociceptive thresholds in laboratory animals, with von Frey filaments and the Hargreaves’ hot plate being some of the instruments most frequently used to measure mechanical and thermal thresholds, respectively (Chaplan et al., 1994; Hargreaves et al., 1988).

For a particular QST technique or protocol to be reliable it needs to meet standards of repeatability and sensitivity. Repeatability can be defined as the level of agreement between measurements from a particular animal obtained by tests separated by short periods of time. Repeatability can be adversely affected by the animal learning to react to the stimulus before it becomes noxious (Chambers et al., 1990) and can depend on the precise location of the site that is stimulated (Haussler et al., 2007). Sensitivity refers to the ability of the method to detect changes in the measured quantity. Several studies have shown decreased mechanical nociceptive thresholds in animals following surgery or in animals with chronic pain, while other studies have shown increased nociceptive thresholds after analgesic treatment (KuKanich et al., 2005; Lascelles et al., 1997; Lascelles et al., 1998; Ley et al., 1995; Slingsby et al., 2001). Another important aspect regarding the development of QST methods is that they have to be tailored to the behavioural characteristics of the species they are intended for. For example, a QST device that was attached to the animal's limb using a cuff was successfully used in sheep, cattle and horses, but proved to be not suitable for juvenile pigs, requiring the development of an alternative method (Sandercock et al., 2009).

It remains to be determined if any QST techniques are sensitive and robust enough to be clinically useful in animals as a means of monitoring disease progression or treatment efficacy. A number of factors can influence QST measurements. Mechanical nociceptive thresholds differ between tissues and body regions. For example, horses showed lower thresholds when measured on soft tissue than those measured on bony landmarks and thresholds were also lower over spines of cervical vertebrae than over those of the lumbo-sacral region (Haussler and Erb, 2006). Additional factors potentially affecting QST measurements are sex, height, weight and age, which is particularly relevant when QST measurements from clinical cases are compared with reference measurements made from control animals (i.e. those not in pain). It is important to appreciate that QST techniques provide measures of activation of nociceptor pathways, but not conscious perception of pain. However, they are important

tools, since demonstration of such activation is good evidence that pain may be experienced.

1.11.3 Behavioural quantification

Pain evokes changes in the behaviour of animals and behavioural assessment is crucial in the evaluation of pain in the veterinary patient (Hansen, 1997). Behavioural pain responses are aimed at enhancing the animal's chances of survival. The simplest type of behavioural response to a painful stimulus is an automatic response or withdrawal reflex. Other responses are aimed at protecting the site of injury (e.g. guarding behaviour), minimising the pain, and promoting healing. Often, pain can be manifested as aggression, even in animals that have not displayed aggressive behaviour previously. The recognition of pain behaviours in animals will depend on a number of factors relating to the animal assessed, the observer making the assessment and the environment where the assessment is taking place. Animal-dependent variables include species, or even strain, sex, weight, age, social dominance, previous conditioning and overall health (Hardie, 2000). Observer-dependent variables include knowledge of the species (or even the individual's) behaviour, observational skills and attitudes towards pain in animals or in that particular species (Hardie, 2000).

The effects of chronic pain on animals' behaviour are more subtle and are difficult to both identify and quantify. Questionnaire based owner-reported changes in behaviour have been shown to provide a reliable tool for measuring chronic pain in dogs (Wiseman-Orr et al., 2006) but this approach might not be suitable for other species.

In laboratory animal models of chronic pain, there appears to be activation of the HPA axis and 'depressive-like' symptoms similar to the responses produced by an inescapable stressor (Blackburn-Munro and Blackburn-Munro, 2001). A recent study in mouse models found that chronic pain caused long-term anxiety that persisted beyond the reversal of the hypersensitive period associated with the pain (Narita et al., 2006), and owner-reported behavioural changes have already shown that dogs suffering from chronic pain exhibit altered attitude and demeanour that might be indicative of depression or anxiety (Wiseman et al., 2001).

The affective component of the pain experience is acknowledged in a wide variety of animal species in terms of the neurobiology and the responses observed, but its measurement still remains a problematic. A novel approach that might prove useful for assessing the affective component of pain in animals could be through measurement of cognitive biases. In humans, emotions are associated with biases in information processing and research has revealed a relationship between negative affective states and pessimism and risk-aversion (e.g. Eysenck et al., 1987). Similar cognitive biases have been identified in rats and starlings, showing that stressed animals behave "pessimistically" when presented with an ambiguous stimulus (Brilot et al., 2010; Enkel et al., 2010; Harding et al., 2004). Although cognitive bias testing has not yet been shown to provide a reliable and sensitive method for assessment of chronic pain in animals, this methodology may well hold potential to investigate the affective component of chronic pain in animals.

1.12 Overview of experimental work described in this thesis

This PhD research project has two components. Firstly, a translational research component that aims to validate a novel QST method for assessing mechanical nociceptive thresholds in horses and establish whether neuropathic changes contribute to the chronic pain state observed in equine laminitis. The second component reports the discovery and investigation of an endogenous mechanism involving 5-HT_{1B} receptors that facilitates the activation of TRPM8 receptors and is predicted to enhance the analgesic influence they have been previously shown to exert.

Chapter 2: MATERIALS AND METHODS

2.1 Animals

All experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and associated guidelines, and had received ethical approval from the University of Edinburgh ethical committee.

With regard to the use of quantitative sensory testing in horses, it was agreed with the UK Home Office Inspectorate that any pain inflicted was of limited severity and of short duration, that the results were of potential benefit to the horses being tested, and that the studies did not need to be licensed as scientific procedures under the Animals (SP) Act (1986).

2.1.1 Horses (laminitic and control)

Seven horses suffering from refractory (chronic) laminitis for more than 2 months were selected, with their owners' consent, from patients referred to the Royal (Dick) School of Veterinary Studies, and from horses under the care of the International League for the Protection of Horses (now World Horse Welfare), in Aberdeenshire. As the laminitic cases were drawn from a clinical caseload, therapy was not discontinued during the measurement period.

Seven normal (control) horses, each one matched as far as possible with one of the laminitic horses for size, age, breed and sex, were selected, with their owners consent, from amongst horses in local riding schools or belonging to members of staff of the Royal (Dick) School of Veterinary Studies.

2.1.2 Rats (chronic constriction injury (CCI) model of neuropathic pain)

Wistar rats (Harlan, UK), weight between 180-250 g, were used in all experiments and were given access to food and water *ad libitum* and housed in accordance with Home Office guidelines. The chronic constriction injury (CCI) developed by Bennett and Xie (1988) was used as a model of neuropathic pain. Prior to surgery, animals were anaesthetised in about a 3% isoflurane/oxygen mixture for the entire procedure (Zeneca, Cheshire, UK); following exposure of the sciatic nerve, proximal to the trifurcation at mid-thigh level, four loose

ligatures of chromic cat gut (SMI AG, Hunningen, Belgium) were tied around the nerve, separated by 1 mm from each other. Following suturing, animals were allowed to recover in a protected and warm environment. CCI rats consistently developed mechanical and thermal reflex sensitisation, which peaked between days 9 and 16 post-surgery, when drug-testing or tissue collection were carried out. Development of sensitisation was always quantified behaviourally before carrying out further experiments.

2.2 Quantitative sensory testing

Behavioural reflex responses to noxious mechanical stimuli were measured in horses using a newly developed hoof tester, as described below. In laboratory rats, behavioural reflex responses to punctate mechanical and noxious thermal stimuli were measured. Animals were habituated to the testing conditions before performing tests from which results were recorded. In laboratory rats, a minimum of 3 tests was performed for each type of method, in order to establish a consistent baseline before drug application or to measure development of sensitisation prior to tissue collection.

2.2.1 Hoof testing

A feedback-controlled, hydraulic hoof-tester was designed and built by Robert Clark (Consultant Engineer, Roslin, Midlothian) in order to measure hoof compression thresholds (HCTs) in horses. This instrument (Figure 2.1) is powered by a hydraulic motor that pumps fluid at high pressure through flexible lines to a piston and a stimulating probe connected to a load cell. For stimulation, the circular flat surface of the probe (14.0 mm diameter) was applied to the sole of the hoof and its position secured by applying the non-slip “anvil” of the hoof tester on the dorsal hoof wall. A process controller (Eurotherm 3508, Eurotherm, UK) used the output from the load cell to set a maximum (safety cut-off) and permitted the output of the load cell to be logged into an Excel file.

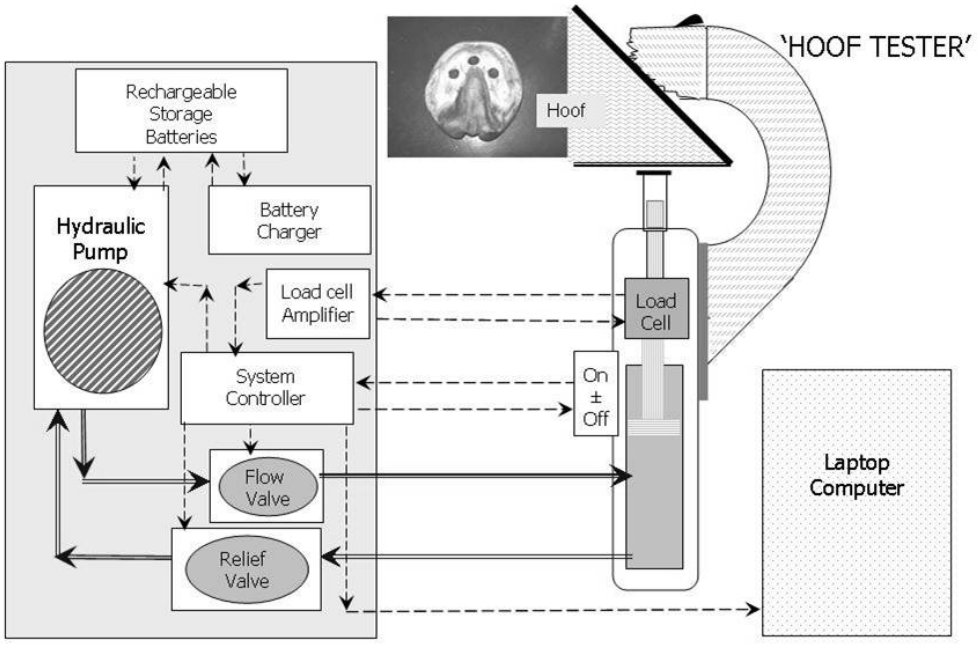

Figure 2.1: *Hydraulically-powered and feedback controlled Hoof Tester. The diagram shows the hand piece, its connections to the control system and to a laptop computer. A hoof with surface marks on the sole is shown to indicate the location of the three sites (Toe, Medial and Lateral) used for testing.*

Testing was carried out in a quiet environment and, prior to testing, the horses were allowed 10 mins to become accustomed to the environment and habituated to the noise produced by the hoof tester. The horses were tested while standing squarely on a firm level surface and were restrained, using a head collar, by an experienced horse handler. All testing was performed after lifting the horse's forelimb and holding it between the observer's thighs, with the sole of the hoof facing up, as is standard practice in farriery.

After cleaning the sole with a wire brush or hoof knife, the flat, circular, surface of the probe was applied to three sites; labelled Toe (half way between the apex of the frog and the white line), Medial (to the apex of the frog) and Lateral (to the apex of the frog) (Figure 2.1).

Application of gradually increasing pressure was started and stopped by a single 'on-off' thumb-switch on the handle of the instrument. HCTs were

detected by the operator at the onset of a withdrawal response of the limb and pressure on the probe was immediately released. This response was generally detected as a sudden change in tone of the flexor muscles of the limb being tested. If the horse did not respond, pressure was automatically released through a relief valve, set to open at 85 kg/cm² to avoid damage. The rate of application and the setting for the pressure relief valve (maximum) were chosen after pilot studies in which the rate, duration and maximum pressure applied to 'normal' horses were gradually increased. Pre- and post-test lameness assessments, at a walk and trot, did not detect any adverse effects.

The pressure exerted by the probe (kg/cm²) was calculated from the force measured by the, in-line, calibrated load cell and the contact area of the probe (1.54 cm²). Pressure during each test was monitored graphically and recorded to an Excel file on a laptop computer.

Horses were tested in three test sessions separated by one week. Within each session, the forefeet of horses were tested twice at the three sites (Toe, Medial and Lateral). Thus, 12 tests were obtained per horse on each session and a total of 36 tests per horse. Eight (8) readings were not successfully recorded due either to operator error or failure to get the horse to cooperate. In total there were 250 measurements from the laminitic group, and 246 from normal animals.

2.2.2 Mechanical allodynia (von Frey filaments test)

Sensitivity to mechanical stimuli was assessed in laboratory rats by measuring the 50% paw withdrawal threshold (PWT) to calibrated Semmes-Weinstein von Frey filaments (Stoelting, Illinois, USA). Animals were placed on a raised mesh grid and covered with a clear plastic box in order to contain them without impeding their free movement. Von Frey filaments were applied in a series of increasing calibres, perpendicularly to the plantar surface of the hind paws until they bent. PWTs were recorded as the pressure (mN/mm^2) required to elicit a withdrawal response to ≥50% of applications. A minimum of 6 applications of each filament to the foot was used to establish the withdrawal threshold. A pressure of 1,609.52 mN/mm^2 was set as the maximum since this force was sufficient to lift the paw.

2.2.3 Thermal sensitivity (Hargreaves' test)

Sensitivity to a noxious thermal stimulus in laboratory rats was assessed by measuring paw withdrawal latency, in seconds (s), in response to an infrared beam (55°C, Hargreaves' thermal stimulator, Linton Instrumentation, Diss, UK) directed to the mid-plantar glabrous surface of the hind paw. Withdrawal responses were only noted when they indicated clear nocifensive behaviour (typically manifested as a "flick" of the paw, sometimes followed by licking) and their latencies were recorded to the nearest 0.1 s. A maximum cut-off latency of 20 seconds and a minimum of 5 mins recovery time between tests were established in order to prevent tissue damage and sensitisation.

2.3 Drug administration in laboratory rats

Drug administration in laboratory rats was carried out by the intrathecal route. Drug effects were assessed by quantification of threshold to noxious stimuli. A minimum of three pre-drug tests were performed to produce a mean baseline value. Testing commenced 15 mins after injection to allow recovery from anaesthesia and avoid a false analgesic effect and tests were repeated every 10 mins for 60 mins for thermal and mechanical stimulation. An n of 6 rats was used for each drug.

2.3.1 Intrathecal application of drugs

Drugs were administered by percutaneous puncture into the L5/6 intervertebral space of briefly anaesthetised (3% isoflurane/oxygen over the course of the injection) in CCI rats, at the peak of behavioural reflex sensitisation, in a volume of 50 µl, using a 0.5 ml syringe with 29-gauge needle (BD Biosciences, Oxford, UK). The vehicle for drug injections was saline with up to 0.5% dimethyl formamide, which has been extensively characterised previously in the laboratory as having no effect on behavioural reflex responses.

In order to investigate the possibility of an interaction between the TRPM8 receptor and the 5-HT_{1B} serotonin receptor, the following drugs were applied

intrathecally: the TRPM8 agonist, icilin (3,4-dihydro-3-(2-hydroxyphenyl)-6-(3-nitrophenyl)-(1H)- pyrimidin-2-one, Bioscience, Bristol, UK 2.5 nmol), the potent, selective 5-HT_{1B} agonist CP 94253 (5-propoxy-3-(1,2,3,6-tetrahydro-4-pyridinyl)-1H-pyrrolo[3,2-b]pyridine hydrochloride, Tocris, 2.5 nmol), the selective 5-HT_{1B} antagonist, SB2242289 (hydrochloride (1'-methyl-5-[[2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]carbonyl]-2,3,6,7 tetrahydrospiro[furo [2,3-f]indole-3,4'-piperidine hydrochloride, Tocris, 100 μM) and the PLD (and PKC) inhibitor, calphostin-C ((1R)-2-[12-[(2R)-2-(benzoyloxy)propyl]-3,10-dihydro-4,9-dihydroxy-2,6,7,11-tetramethoxy-3,10-dioxo-1-perylenyl]-1 methylethylcarbonic acid 4-hydroxyphenyl ester, Tocris, 0.65 nmol).

2.4 Electron microcrographic analysis of nerve morphology

Lateral digital nerves (3 cm long segments) from five horses euthanized on welfare grounds due to refractory laminitis were dissected from the forelimb at the level of the proximal sesamoid bone and fixed for 4 h in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) post-fixed in OsO_4 and embedded in Araldite. Control tissue was collected and treated as above from four horses which had no previous history of forelimb lameness and were euthanised for clinical reasons other than forelimb pathology. 1 μm resin sections of the nerve were stained with Toluidine Blue and three fascicles were chosen at random by bright field microscopy. Ultra-thin (80 nm) sections were stained with uranyl acetate and lead citrate for examination and image capture (done by Steven Mitchell) using a Phillips BioTwin electron microscope (FEI, UK Ltd, Cambridge, UK). Identity-concealed images of randomly selected fascicles (areas ranging between 6,732 and 47,215 μm^2) were analysed using Image Tool 3.0 (UTHSCSA, USA).

In order to investigate any differences between normal and laminitic digital nerves that might reflect oedema and therefore affect the quantification of axon density, the total area of the nerve sections and the percentage of the nerve area occupied by nerve fascicles were calculated. The number and axon diameter of

intact myelinated fibres was calculated as well as the percentage of damaged myelinated fibres, defined as those with a severe disruption of the myelin sheath and/or axonal degeneration. Myelin sheath thickness was measured and *G*-ratio of axons was calculated by dividing the axonal diameter by the total diameter of axon plus myelin sheath. The proportion of A-fibres with continuous Schwann cell cytoplasm (a nerve morphology abnormality previously described by Court et al. (2004) was also calculated.

C-fibres were identified as small diameter unmyelinated fibres, often surrounded when in bundles by Schwann cell cytoplasm. The total number of C-fibres was calculated as well as the percentage of solitary unmyelinated fibres and the number of unmyelinated fibres per Remak bundle.

2.5 Immunohistochemical analysis of protein localisation

2.5.1 Tissue processing for immunohistochemistry

DRG from cervical segments seven (involved in forelimb innervation) and four (not involved in forelimb innervation) from the same horse were obtained post-mortem from five laminitic horses and four control horses for ATF-3 and NPY immunohistochemical localisation and four laminitic horses and four control horses for TRPM8 immunohistochemical localisation. The tissue was embedded in OCT embedding matrix (CellPath plc., Powys, Wales, UK) and fast-frozen in isopentane suspended in liquid nitrogen or dry ice before storing at –80ºC.

2.5.2 Immunohistochemistry procedure

Cryostat sections of DRGs (15 μm) were thaw-mounted on poly-L-lysine slides (BDH Merck Ltd., Poole, UK). Only every sixth section was selected, in order to ensure that measurements were taken only once for each cell. DRG sections were pre-incubated for 1 h (2 h for TRPM8 immunohistochemistry) at room temperature in 0.1 M PBS buffer, pH 7.4, containing 0.2% Triton X-100, 2-4% fish skin gelatin and 10% normal goat serum; and then incubated overnight at 4 °C (or at room temperature for TRPM8 immunohistochemistry) with primary antibodies diluted in the same buffer. For co-localisation of the peptide

NPY, the activating transcription factor ATF-3 or the TRPM8 receptor with the myelinated cell marker neurofilament 200 kDa (NF-200), or either of the unmyelinated cell markers, peripherin or isolectin B4 (IB4) binding target, antisera/lectin were used at the following concentrations: rabbit anti-NPY (1:250; Peninsula Laboratories Inc, Belmont, CA, USA); rabbit anti-ATF-3 (1:300; Santa Cruz Biotechnology, Santa Cruz, CA, USA); rabbit anti-TRPM8 (1:200; Alomone, Jerusalem, Israel); mouse monoclonal anti-NF-200 (1:400 or 1:1000 for co-localisation with TRPM8; clone N52; Sigma); mouse monoclonal anti-peripherin (1:250 or 1:500 for co-localisation with TRPM8; Chemicon International, Harlow, UK); IB4 from *Bandeiraea simplicifolia* (1:400; Sigma). Sections were then washed in buffer and incubated at room temperature for 2 h (3h for TRPM8 immunohistochemistry) in in 0.1 M PBS, pH 7.4, buffer containing 4% fish skin gelatin and 10% normal goat serum with appropriate secondary antibodies: Alexa Fluor 488-labelled goat anti-mouse IgG (1:500), Alexa Fluor 568-labelled goat anti-rabbit IgG (1:1000), Alexa Fluor 488-labelled streptavidin (1:200), Alexa Fluor 488-labelled goat anti-rabbit IgG (1:750) and Alexa Fluor 568-labelled goat anti-mouse IgG (1:750) (all from Molecular Probes Europe BV, The Netherlands).

Finally, sections were washed three times in 0.1 M PBS before cover-slipping with Vecta-Shield (Vector Laboratories, Burlingame, CA, USA). Control sections were processed as above omitting the primary reagents. Observations were made and sections photographed on an Olympus microscope equiped for epifluorescence. For ATF-3 and NPY immunohistochemical localisation, sections were visualised and photographed under a Leica TCSNT confocal microscope (Leica Microsystems GMBH, Germany). All counts of profiles labelled for immunopositive cells were (following blinding) on five or randomly selected sections of DRG from each of the animals in each group. Image analysis was performed with Image Tool and ImageJ (RSB-NIH, Maryland, USA). Results were expressed as the proportion of labelled profiles per total number of single or double-labelled profiles from all sections, 95% confidence intervals (CI) are indicated.

2.6 Western blot analysis of proteins

2.6.1 Tissue removal and processing for Western blot

DRG and spinal cord tissue from L4-L6 was collected from live animals under deep anaesthesia in 3% isoflurane/oxygen. A laminectomy was performed to expose the spinal cord, and tissue was dissected out. Spinal cord from CCI rats was hemisected into ipsilateral and contralateral sections. Ipsilateral and contralateral DRGs were also collected separately. Naïve tissues were collected from the same anatomical region but without hemisecting the spinal cord and with the left and right DRGs being pooled together. In order to minimise degradation of tissues, these were collected onto ice-cold foil, weighed, and promptly homogenised in 20 volumes of ice-cold Laemmli lysis buffer (e.g. 1 ml of buffer to 0.05 mg of tissue) (85 % Tris buffer (tris-hydroxymethylaminoethane, 50 mM, pH 7.4, Sigma Chemical Co., UK), 5 % mercaptoethanol (Sigma), and 2 % sodium dodecyl sulphate (SDS, Sigma)). Following homogenisation, whole cell lysates were heated to 100 ºC for 5 mins and then centrifuged for 10 mins at 12,000 g and 4 ºC. Supernatants were aliquoted and stored at –20ºC.

2.6.2 Western blot procedure

Western blotting was used to detect changes in protein levels in DRG or spinal cord tissue from CCI and naïve rats.

Proteins were separated by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) using the NuPage XCell *Sure*Lock^{TM} Minicell gel electrophoresis system (Invitrogen, Paisley, UK). Samples (5-8 µl) were mixed with 1-2 µl loading buffer (0.04 % w/v Bromophenol Blue in glycerol) and loaded into wells on 4 – 12 % Bis-Tris NuPage gels (Invitrogen). Pre-stained standard molecular weight proteins (SeeBlue Plus, Invitrogen) were run alongside as a guide. Samples were electrophoresed using appropriate MOPS running buffer (NuPage, Invitrogen) under a 200 V potential difference. Proteins were transferred to a polyvinylidene difluoride Immobilon-P^{SQ} membrane (Millipore, Watford, UK) at 30 V in transfer buffer (5 % NuPage transfer buffer, 10% methanol) for 90 mins. Efficacy of transfer and protein loading was

assessed by staining membranes with Coomassie blue (0.1 % in 30 % methanol, 10 % acetic acid, GE Healthcare Ltd., UK). Following de-staining of the Coomassie blue with 30 % methanol/10 % acetic acid in distilled water, membranes were incubated in appropriate blocking buffers to block non-specific activity (Table 2.1). Membranes were then washed and incubated with appropriate primary antibodies as described in Table 2.1. Blots were also probed with antibody for the ubiquitous housekeeping enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Chemicon) to enable protein level normalization. Following primary antibody incubation, membranes were washed and incubated with peroxidase-linked secondary antibodies: goat anti-rabbit (Cat No. AP182P, 1:7,500; Chemicon), goat anti-mouse (Cat No. AP192P, 1:10,000; Chemicon) or donkey anti-goat/sheep (Cat No. AB324P, 1:10,000; Chemicon) made up in blocking buffer, for 50 mins at room. Staining was detected by peroxidase-linked chemiluminescent substrate and film development (ECL; Cell Signalling, MA, USA, Hyperfilm; GE Healthcare UK Ltd., Bucks, UK). Following quantitative densitometry, using Adobe Photoshop, arbitrary grey scale values for protein bands of interest were calculated as a percentage of that for GAPDH.

Antibody	Source	Block	Dilution	Incubation
Rabbit polyclonal anti-TRPM8 Cat No. ACC-049	Alomone (Jerusalem, Israel)	5% non-fat dry milk in PBS 0.1M/ Tween-20 (overnight at 4º C)	1:200	1 h at room temperature in block solution
Goat polyclonal anti-$5HT_{1B}$ Cat No. sc1460	Santa Cruz, Santa Cruz, CA, USA	3% non-fat dry milk in TBS 0.1M (overnight at 4º C)	1:100	1 h at room temperature in block solution
Mouse monoclonal anti-GAPDH Cat No. MAB374	Chemicon, Harlow, UK	5% non-fat dry milk in PBS 0.1M/Tween-20 (overnight at 4º C)	1:750	1 h at room temperature in block solution
Rat monoclonal anti-HA-Peroxidase (clone 3F10) Cat No. 12013819001	Roche Diagnostics (West Sussex, UK)	5% non-fat dry milk in PBS 0.1M/ Tween-20 (overnight at 4º C)	1:300	1 h at room temperature in block solution

Table 2.1: *Antibodies used for Western blotting.*

2.7 Cell culture

2.7.1 Dorsal root ganglion neuron culture

Dorsal root ganglion neuron culture was carried out by Andrew Allchorne as follows. Coverslips were coated with poly-D-lysine (Sigma). DRG from adult rats (normally 10-14 per animal) were harvested and dissected into Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% FBS and Penicillin/Streptomycin (Invitrogen). After centrifuging for 3 mins at 1,000 g, medium was removed and collagenase (5 mg/ml) and dispase (1 mg/ml) (Roche) added and incubated at 37ºC for 2 h. Following centrifugation at 1000 g for 5 mins, the supernatant was discarded and the precipitate was digested in 0.25% trypsin (Invitrogen) at 37ºC for 8 mins. Then, 0.25% trypsin inhibitor (Invitrogen) was added and preparation was centrifuged at 1,000 g for 5 mins. The supernatant was removed and the pellet was washed with DMEM/10% FBS and re-centrifuged at 1,000 g for 5 mins. After removing the supernatant, DRGs were re-suspended in DMEM/10% FBS. Following the addition of DNAse I (Sigma), DRG preparations were triturated to dissociate to a single cell suspension. This suspension was then filtered through a 15% BSA (Sigma) solution by centrifugation at 1,000 g for 10 mins and then re-suspended in DMEM/10% FBS. The cell suspension was then purified by passage through a percoll solution (1ml HBSS (x10), 1ml 0.35% $NaHCO_3$, 5 ml distilled H_20, 30µl 1N HCl and 3ml Percoll (1.13g/ml solution; Sigma) at 1,000 g for 10 mins at 4^oC.

The lower percoll layer was re-suspended in DMEM/10%FBS and centrifuged for 10 mins at 1,000 g. The cell pellet was then re-suspended in plating medium (GIBCO Neurobasal (Invitrogen, supplemented with GIBCO B-27 Penicillin/Streptomycin (1:100), L-glutamine (200mM, Invitrogen) NGF (50nm/ml; BMB) and Ara-C (Cytosine β-D-arabinofuranoside hydrochloride, 1:1000; Sigma C-6645) in an appropriate volume for the number of cells, as assessed by using a haemocytometer. Following plating, cells were incubated at 37ºC in an atmosphere containing 0.5% CO_2 and used the following day for calcium imaging.

2.7.2 Stable TRPM8 HEK293 culture

A line of HEK293 cells stably expressing human TRPM8 was generated by other laboratory members. 24-well plates (Greiner) were coated with fibronectin (0.01mg/ml; Sigma) in distilled water and allowed to air-dry in the fume-hood for 45 minss.

Stock cultures of TRPM8-expressing HEK293 cells were trypsinised with Tryple Express (Invitrogen) for 1 min and incubated at 37°C for 6 mins. 8ml of complete DMEM without Phenol Red (Invitrogen), containing 10% foetal calf serum (Harlan/Sera Lab, UK) were then added to the flask, and cells were counted before plating out and subculture. Final volume was made up using complete DMEM without Phenol Red. Cells were plated out at a density of 1.5 x 10^5 cells/well (1ml/well) and incubated at 37°C. 60-80% confluency was reached after approximately 24 h.

2.7.3 Transient cell transfection

In order to investigate the possible *in vitro* interactions between the TRPM8 receptor, the 5-HT_{1B} receptor and PLD1, stable TRPM8 HEK293 cells were transfected with FLAG-tagged 5-HT_{1B} receptor and HA-tagged PLD1 cDNA (constructed by Eve Lutz and Mike Frohman, respectively) in pcDNA3.1 expression plasmid or empty vector. FuGENE6 transfection reagent (Roche) was added in a ratio of 3:1 to cDNA and incubated at room temperature for a minimum of 5 mins before transfection using the FUGENE 6-cDNA complex according to the manufacturer's recommended protocol.

Cells were then grown for 48 hours at 37°C in a 5% CO_2, 95% O_2 humidified environment.

2.8 Immunoprecipitation

Cells were quiesced for 4 h in 10 ml of DMEM per 175 cm^2 flask. Cells were then homogenised in IP buffer (20% glycerol, 1% CHAPS, 1% sodium deoxycholate, 1mM sodium orthovanadate in HEPES 20 mM/NaCl 150 mM buffer, pH 7.4), with 1:100 protease inhibitor cocktail 3 (Calbiochem, UK).

Flasks were kept on ice for 30-45 mins and then cells were scraped, removed and centrifuged at 10,000 g for 10 mins at 4ºC to pellet insoluble materials. Supernatants were then decanted and pre-cleared with Protein G-Sepharose (40 µl/ 2ml supernatant; Sigma, UK) while rotating at 4ºC for 45 mins, in order to remove any proteins that non-specifically bind to the beads. Following a pulse-spin the supernatant was removed and Protein G-Sepharose beads (40 µl/ml) added together with the relevant antibody. For immunoprecipitation of the FLAG-tagged 5-HT_{1B} receptor, a mouse monoclonal anti-FLAG M2 antibody was used (4.32 µl/ml; Sigma-Aldrich). For TRPM8 immunoprecipitation, an anti-TRPM8 rabbit antibody was used (7.5 µl/ml; Alomone). For immunoprecipitation of HA-tagged PLD1, a mouse monoclonal anti-HA antibody (clone 12CA5) was used (5 µl/ml; Roche). As controls, non-immune IgG of the same species as the antibody were used for immunoprecipitation. Primary antibodies and non-immune IgGs were incubated with samples overnight at 4ºC. Samples were then centrifuged and the supernatants discarded. The beads were then washed three times with ice-cold IP buffer after which all liquid was removed and a volume of Laemmli buffer equal to that of beads was added to each tube. The samples were then thoroughly mixed and heated at 70ºC for 10 mins and then either used immediately or stored at -20 ºC. Binding partners within the complex of interest were detected by Western blot, (as described in 2.6.2) running 15-20 µl of sample (5-10 µl for totals) and 2 µl of loading buffer.

2.9 Calcium fluorometry

Stable TRPM8 HEK293 cells with appropriate cDNA transfections were cultured in 1ml medium per well of a Greiner 24-well clear flat-bottomed plate for 48-72 h before assay (as described on 2.7.2 and 2.7.3). On the day of assay 700 µl medium was removed from each well prior to the addition of 100 µl of calcium indicator dye solution (Calcium 4 Dye, Molecular Devices, CA, USA), pre-warmed to 37^{o}C, to the remaining 300 µl. Calcium 4 Dye was dissolved in concentrated form in dimethylsulphoxide (Sigma D2650) and aliquoted at -20^{o}C.

When required an aliquot was made up in 2xSPZ medium according to the manufacturer's instructions at a concentration equivalent to 4 μM. 2x SPZ consists of divalent-free Hanks' Balanced Salt Solution (Gibco 14175) with 1.8 mM Ca^{2+} (900 μl of 1M volumetric standard $CaCl_2$; BDH 19046) and 0.8 mM Mg^{2+} (82 μl of 4.9 M volumetric standard $MgCl_2$ (Sigma Diagnostics 104-20)) with 25 mM HEPES pH 7.40 (12.5 ml of 1 M HEPES (Sigma H3375) adjusted to pH 7.40 with NaOH) per 500 ml containing 500 mM (±) sulphinpyrazone (1 ml of 0.25 M solution of sulphinpyrazone, Sigma S9509) in N,N-dimethylformamide (Sigma D4551) per 500 ml.

Plates were incubated at 37°C for 45 mins in the dark in a 95% O_2 / 5% CO_2 atmosphere. Plates were then transferred to a shallowly immersed platform in a water bath at a holding temperature of 28°C for 5 mins to ensure temperature equilibration. A 50 μl aliquot of 250 mM HEPES pH 8.50 was then added to each well (which effectively reversed the acidification of the medium due to metabolism and the blockade by sulphinpyrazone of the non-specific anion export carrier, restoring a pH of 7.4) and incubation was continued for a further 2 mins at 28°C.

With the plates still at 28°C, compounds were added in volumes from 1-4 μl per well from generally 100x concentrated solutions in dimethylformamide yielding final vehicle concentrations from 0.25 – 1 % (which were documented in other studies in the lab to have no effect alone on Ca^{2+} regulation).

Intracellular Ca^{2+} was measured using a Varioskan Flash fluorometric plate reader (Thermo Laboratory Instruments). The typical standard plate layout contains triplicate determinations of no compound (or control treatment) and five measurements following TRPM8 agonist administration with four different additional conditions being applied in each case. Ten readings from each well were taken 30 sec apart at excitation 488 nm, emission 519 nm in bottom-reading mode for the plate reader. Fluorescence reflecting intracellular Ca^{2+} levels (arbitrary units) was recorded using the SkanIt software (Thermo Fisher Scientific, Waltham, MA, USA). Data was analysed as percentage-increase over baseline control, and plotted as arbitrary relative fluorescence units.

The following drugs were used: the TRPM8 agonist icilin (0.25 μM, Tocris), the TRPM8 selective channel bolcker AMTB (N-(3-aminopropyl)-2-[[(3-methylphenyl) methyl]oxy]-N-(2-thienylmethyl)benzamide hydrochloride salt, Tocris 3989), the 5-HT_{1B} agonist CP 94253 (2 μM; Tocris) , the 5- HT_{1B} selective antagonist SB224289 hydrochloride (1'-methyl-5-[[2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]carbonyl]-2,3,6,7tetrahydrospiro[furo [2,3-f]indole-3,4'-piperidine hydrochloride, Tocris, 100 μM), the PLD inhibitors CAY10593 (also known as VU0155069, 0.5 μM; Cayman Chemicals, Michigan, USA), halopemide (4 μM, Cayman), raloxifene (25 μM; Tocris) and calphostin C (2.5 μM; EnzoBioscience); the PKC inhibitor BIM1 (2.5 μM; EnzoBioscience); the Phosphoinositide-3-kinase (PI 3-K) inhibitor, wortmannin (30 μM; Tocris), the synthetic PLC activator m-3M3FBS (2.5 μM, Tocris) and the PLC inhibitor U-73122 (10 μM; EnzoBioscience).

2.10 Calcium imaging of DRG cells

Ratiometric calcium imaging was performed with FURA-2/AM dye (Invitrogen) and analyzed using Simple-PCI software (Compix Inc.). Cells were loaded with 3.5 μM Fura-2/AM (Invitrogen, Paisley, UK) in DMEM supplemented with 10% pluronic acid (Invitrogen) for 20 mins. Following washing, coverslips were placed in a recording chamber containing 2.5 ml of DMEM. An Olympus BX50WI microscope was used (20x magnification objective lens). Pairs of images were collected every 5 seconds at alternating exposures of 340 nM and 380 nM using a Photonics LPS150 monochromator and imaged using a Hamamatsu ORCA-ER CCD camera.

Coverslips were superfused with DMEM buffer at approximately 0.5 ml/min. Drugs were applied via a gravity driven perfusion system that allowed rapid exchange of solutions. Temperature was maintained at 28.5°C for the duration of the experiment.

Following selection of a suitable field of view, recording was initiated. The effects of drugs were compared through paired permutations. After a baseline period of 5 mins the first drug treatment was applied for 10 mins, followed by a

10 mins wash-out period and the second drug treatment. The following drugs were used: icilin (1.25-2.5 μM), CP 94253 (3 μM), calphostin C (2.5 μM) and BIM1 (2.5 μM).

For analyisis, following subtraction of background fluorescence, the ratio of fluorescence at 340 nm and 380 nm was calculated. A response was designated as an increase in fluorescence ratio from baseline consistent with the timing of application. Only icilin-responsive cells were selected for the purposes of quantifying responses to further treatments. Area under the curve was determined for the response curve for each of the paired treatments using SigmaPlot.

2.11 Statistics

Statistical analyses were carried out as specified in each case below. In all cases $p < 0.05$ was taken to indicate statistical significance, and degrees of freedom associated with any tests are denoted by subscripts.

2.11.1 EM and ATF-3/NPY immunohistochemistry analysis

Differences in percentages of damaged A-fibres, A-fibres with continuous Schwann cell cytoplasm and solitary unmyelinated fibres between laminitic and control horses were analysed using simple logistic regressions. Axon diameter of myelinated and unmyelinated fibres and myelin sheath thickness data comprised repeated measures, therefore, linear mixed effect models were used to determine any difference in these parameters between laminitic and control horses. Multiple measurements per horse were also taken of the number of fibres per Remak bundle but as the data were integers, differences in the number of fibres per Remak bundle were analysed using generalised linear mixed-effect models with Poisson errors. Differences in the total nerve area occupied by fascicles between control and laminitic horses were investigated using a Student's *t*-test, and differences in the percentage of nerve area occupied by fascicles between normal and laminitic horses by general linear models with binomial errors. Mann-Whitney non-parametric tests were used to analyse fibre density. All analyses

were carried out in S-PLUS 6.0 (Insightful, Seattle, USA) and SigmaStat 2.03 (SPSS Inc., USA).

2.11.2 Hoof tester data analysis

Expert advice on this evaluation was taken from Iain McKendrick (BioSS). Preliminary statistical analysis to investigate differences in hoof compression thresholds between normal and laminitic horses was carried out using a Linear Mixed model (Brown and Prescott, 1999), treating Pressure as the response variable; Group (laminitic or control) and the other variates: Session (1, 2 or 3), Leg (left or right), Site (toe, medial, or lateral) and Test (1^{st} or 2^{nd}) and all interactions nested within Group, fitted as fixed effects; and a factor encoding the sample matching (Matching), its interaction with Group (which is statistically equivalent to fitting an individual horse effect) and all possible interactions of the latter term with the other fixed effects, included as random effects. The fixed effects were progressively removed in a stepwise-type process until only statistically significant factors remained.

In order to better appreciate the sources of variation, data for the two groups were separately analyzed using Linear Mixed models which treated all relevant factors and their interactions as random effects. The estimated variance components are used to summarise the sources of variability in the data. The sixteen estimated random effects can be found in Table 3.2. Parsimonious models were also produced, by excluding components that were not associated with a statistically significant improvement in model fit, as quantified by the statistical deviance. The values of the mean and variance components for each group of horses were used, in statistical models, to allow prediction of the benefits of reducing different sources of variability (errors) and prediction of the numbers of animals and tests that would be required to show treatment effects of various sizes.

To assess the repeatability of the method, intra-class correlation coefficients were determined by calculating the ratio of population variability to the total variability estimated from the final fitted model. All mixed modeling was carried out using Genstat 10^{th} edition (VSNi, Hemel Hempstead, UK). Intra-class correlation coefficients, the sensitivity, specificity and area under the

Receiver-Operator Curve were estimated parametrically using these variance components; calculations were carried out in Excel 2003.

2.11.3 QST data analysis

Behavioural data are stated as mean ± standard error of the mean (SEM) and p values <0.05 are considered significant. Parametric statistical tests were used to analyse data generated from the Hargreaves' thermal test. Differences in thermal sensitivity between the paw ipsilateral to nerve injury and the contralateral paw were analysed using Student's t-test. Effect of drug treatment was analysed by One-Way ANOVA followed by Dunnett's post-hoc multiple comparisons test. The equivalent non-parametric tests for mechanical sensitivity were Wilcoxon rank test for ipsilateral: contralateral differences, and Friedman Repeated Measures ANOVA followed by Dunn's test for drug effects.

2.11.4 Western blot analysis

Western blot densitometric values were compared by One-Way ANOVA.

2.11.5 TRPM8 immunohistochemistry

Double labelling cell counts were analysed by One-Way ANOVA.

2.11.6 Calcium fluorometry analysis

Mann-Whitney U tests were carried out to determine differences between each drug and their control treatment. Analysis was performed in GraphPad Prism, version 5.02 for Windows. Relative fluorescence was plotted as mean ± SEM.

2.11.7 Calcium imaging analysis in DRG cells

Areas under the curve for paired-drug treatments were calculated compared using a paired-t test or its non-parametric equivalent, the Wilcoxon test.

Chapter 3: NEUROPATHIC CHANGES ASSOCIATED WITH EQUINE LAMINITIS PAIN

3.1 Introduction

3.1.1 Impact of laminitis on equine welfare

Equine laminitis is a common cause of lameness involving one or more feet (Dyson, 2003); the forefeet being more frequently affected presumably because the forequarters support about 65% of the body weight (Pollitt, 2000). This disease is characterised by the disruption of the dermo-epidermal bond between the distal phalanx and the inner hoof wall, which may result in the displacement and "sinking" of the distal phalanx within the hoof capsule causing structural damage, unrelenting pain and a characteristic lameness (Kyaw-Tanner and Pollitt, 2004).

The importance of this disease as a major concern for the equine industry and its impact on equine welfare has been historically acknowledged. One of the earliest descriptions of equine laminitis was written by the Greek philosopher, Aristotle (384-322 BC), although he referred to it as "barley disease" (cited in Wagner and Heymering, 1999). In the nineteenth century, William Percivall, the British cavalryman and author of the book *Hyppopathology-Lameness in the Horse* (1871) stated that, of all the conditions affecting the horse, "there is no one in which his pain, while poignant to the extreme, is apt to be so protracted" as equine laminitis.

Laminitis is a relatively common disease. A study identified a prevalence of 7.1% in the UK (Hinckley and Henderson, 1996) and a survey carried out in the USA found that 13% of horse owners/operations had reported laminitis in their horses over the previous year (U.S.D.A., 2000).

A further factor contributing to the impact of laminitis on equine welfare is that no therapeutic regime is able to arrest or prevent its onset and the developmental phase of the disease is not clinically recognisable (Bailey et al., 2004; Pollitt et al., 1998). Finally, pain associated with laminitis can be difficult

to control using conventional anti-inflammatory agents and euthanasia on welfare grounds is not uncommon (Herthel and Hood, 1999; Swanson, 1999).

3.1.2 Structure of the laminar structures of the inner-hoof wall

The innermost layer of the hoof wall and bars of equines, known as the *stratum lamellatum* (layer of leaves), comprises 550-600 primary epidermal lamellae (PEL) which project from its surface in parallel rows. Each PEL is further divided, increasing its surface area, into 150-200 secondary lamellae (Pollitt, 2004). The distal parts of primary and secondary lamellae are orientated towards the distal phalanx and interdigitate with the primary and secondary dermal lamellae. The dermo-epidermal interface is comprised by the basement membrane, a sheet of extracellular matrix, which provides a structural bridge, attaching the basal cells of the lamellar epidermis and the connective tissue fibrils that arise from the dorsal surface of the distal phalanx (Pollitt, 2004). During the developmental and acute phases of laminitis, degradation of the basement membrane occurs, with loss of the attachment between epidermal and dermal lamellae (Figure 3.1). Subsequent structural damage may take place, leading to the rotation and "sinking" of the pedal bone within the hoof capsule. Radiographic measurement of this displacement (Figure 3.2) has been reported to be a significant prognostic parameter of the disease (Cripps and Eustace, 1999).

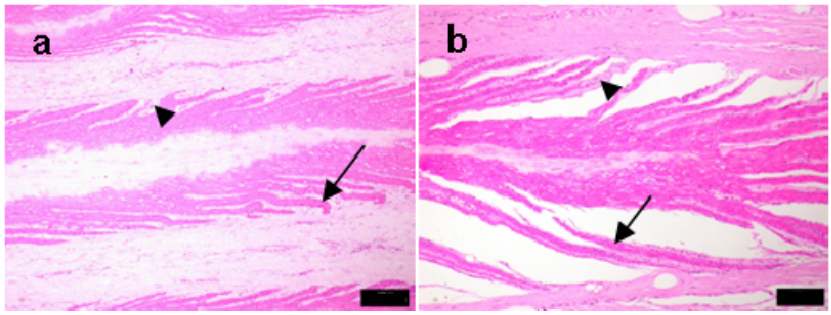

Figure 3.1: *Haematoxylin/Eosin stained histological sections of (a) the intact lamellar structures in a normal horse showing the normal interdigitating of dermal (arrowhead) and epidermal (arrow) laminae 10x. And (b), showing the disruption and separation of the secondary epidermal (arrow) and secondary dermal (arrowhead) laminae in acute laminitis 10x. Scale bars= 100 µm. (From Jones et al., 2007).*

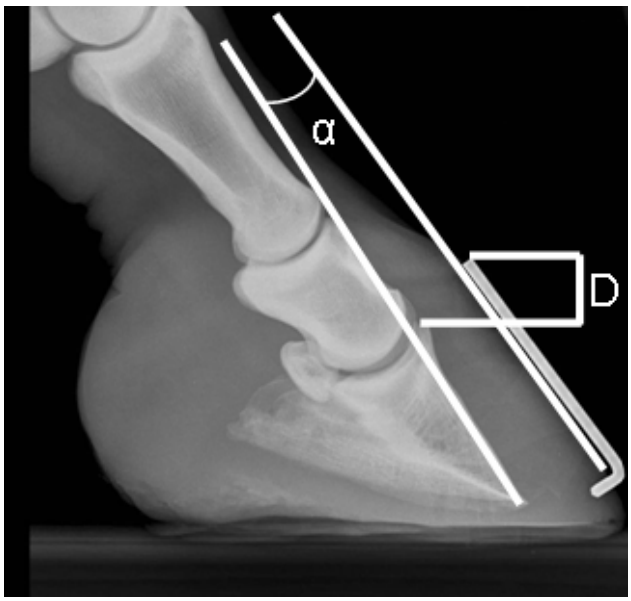

Figure 3.2: *Latero-medial radiograph of equine digit of a laminitic horse showing rotation and vertical displacement (''sinking'') of the third phalanx. Radiographic measures include displacement (D) which is the distance (mm) between the proximal limit of the dorsal hoof wall and the extensor process of the distal phalanx, and the rotation angle (a) which is the angle between the dorsal surface of the distal phalanx and the dorsal surface of hoof wall. (From Jones et al., 2007).*

3.1.3 Pathophysiology of equine laminitis

Despite a wealth of investigative effort, the pathophysiology of equine laminitis has not been fully elucidated. Most laminitis cases occur in horses and ponies kept at pasture and the disease has been linked to the non-structural carbohydrates (particularly fructans) that increase in the pasture at particular times of the year associated with bright sunshine during the day and cool nights (Geor, 2009; Menzies-Gow et al., 2010). Laminitis has also been related to predisposing factors in horses, such as insulin resistance and hyperinsulinemia (Nourian et al., 2009).

Digital blood flow disturbances seem to be fundamental in the pathophysiology of laminitis, with histopathological changes in the laminar structures suggestive of ischaemia-reperfusion injury (Hood et al., 1993). It has been proposed that these changes could be induced by toxic compounds produced in the hindgut either by bacterial fermentation of carbohydrates or from the death or adaptive metabolism of other bacteria, and then released from the intestine into the blood stream. Three types of trigger factors, with possible

cross-talk between their pathways, have been thus suggested: amines, endotoxin and exotoxins (Bailey et al., 2004).

Bacterial fermentation of carbohydrates in the hindgut results in the production of monoamines. Indeed, it has been shown that cecal contents collected from horses fed spring/summer grass has 2 to 3-fold higher concentrations of some mono- and di-amines than those harvested from animals fed with winter grass or hay (Bailey et al., 2003). Additionally, *in vitro* studies have revealed that the digital arteries of horses are 20-30 times more sensitive to the vasoconstrictor effects of serotonin than facial or tail arteries (Bailey and Elliott, 1998). Tryptamine, a cecum-derived amine, causes vasoconstriction through direct activation of serotonin receptors, with a marked selectivity for digital veins (Elliott et al., 2003).

The increase in lactic acid produced by fermentation of carbohydrates in the hindgut results in changes in the microbial/bacterial population that could lead to the release of exotoxins (Mungall et al., 2001). Also pH reduction could result in bacterial death and the release of endotoxins that would then enter the blood stream and act on the digital vasculature. However, the role of endotoxins in the pathogenesis of equine laminitis has been disputed. Studies have given conflicting results in reporting levels of plasma endotoxins in carbohydrate-overload models of equine laminitis (Hood, 1995; Sprouse et al., 1987). Moreover, endotoxin administration has failed to induce laminitis (Hunt et al., 1990), questioning a direct role of endotoxins in the etiology of laminitis.

Equine laminitis is characterised by disruption of the lamellar basement membrane and its attachment to the epidermal basal cells (Pollitt, 1996; Pollitt and Daradka, 1998). The lamellar pathology of laminitis has been associated with the activation of matrix metalloproteinases (MMPs) (Johnson et al., 1998; Pollitt et al., 1998). MMPs are a group of zinc-dependent enzymes that degrade extracellular matrix and basement membrane and are involved in the physiological remodelling of the lamellae as the hoof grows (Pollitt, 1999). Although it has not been fully elucidated what trigger factors are involved in naturally occurring laminitis, *in vitro* studies have shown that bacterial exotoxins

can lead to MMP activation (Mungall and Pollitt, 2002). Additionally, inflammatory mediators (like interleukin-1β) can lead not only to the release of vasoactive mediators but to MMP activation, further giving support to the claim that the vascular and enzymatic theories of laminitis pathogenesis are not mutually exclusive (Moore et al., 2004).

3.1.4 Sensory innervation of the equine digit

Two types of sensory receptors have been identified in the equine foot. Lamellated corpuscles, similar to Pacinian corpuscles, found primarily in the solar dermis of the heel, are low-threshold mechanoreceptors, which transmit their input via rapidly conducting, myelinated A-fibres (Bowker et al., 1993). Additionally, naked nerve endings immunoreactive to CGRP and sensory neuropeptides such as substance P (SP), neurokinin A (NKA) and peptide histidine-isoleucine (PHI) were detected in the dermis of the dorsal hoof wall and sole (Bowker et al., 1995). Immunoreactive nerve fibers were observed to extend into the primary and secondary dermal laminae and, in the palmar regions, they extended through the digital cushion and surrounding connective tissue into the dermal tubules of the solar dermis (Bowker et al., 1995). Nerve fibres containing CGRP are associated with nociception and they transmit their input via slowly conducting C fibres (Schmidt, 1981). Some of these unmyelinated nerves might also serve an efferent function in the control of blood flow within the foot (Bowker et al., 1995).

3.1.5 Pain assessment in laminitic horses

Accurate evaluation of pain and associated lameness in laminitic horses is of fundamental importance to equine welfare and effective veterinary management of the equine patient. In a clinical context, lameness is typically assessed subjectively by veterinarians through observation of the horse at rest and at exercise, and thorough clinical examination. Such subjective assessment is not reproducible and is highly influenced by the experience and expertise of the clinician. Lameness, particularly in milder forms, can be very difficult to evaluate on the basis of clinical examination alone by even highly experienced clinicians (Schatzmann, 2000). In an attempt to standardise pain assessments and

make them more systematic and less "subjective" a number of scoring systems have been put forward. The Obel Score (Obel, 1948) and the Clinical Grading System developed by Taylor et al. (Taylor et al., 2002) are two simple descriptive scales (SDS) developed specifically for evaluation of equine lameness. Simple Descriptive Scales consist of a number of expressions used to describe different levels of pain intensity, such as "no pain", "mild", "moderate" and "severe". Each descriptor is assigned an index value (generally from 0 - 5), which becomes the pain score for the animal. Although Obel score and CGS are extensively used to assess lameness in clinical practice and to evaluate response to therapy, the performance of these scales has not been thoroughly validated (Keegan et al., 1998). Studies that investigated subjective scales used to assess lameness by comparing scores with objective measurements from force plates or gait-analysis identified significant inter-observer variability and poor agreement with objective assessments (Hood et al., 2001; Keegan et al., 1998; Peloso et al., 1993; Silver et al., 1983; Taylor et al., 2002). A recent study evaluating subjective scales for the assessment of laminitis pain has identified suboptimal levels of inter-observer reliability and has highlighted the role of experience when using these scales (Vinuela-Fernandez et al., 2010).

Recognising the inherent limitations of subjective techniques for evaluating equine lameness, quantitative evaluation systems (including behavioural quantification and the use of calibrated hoof testers) offer potentially superior techniques for the assessment of laminitis pain.

Foot-lifting has been identified as a reliably quantifiable behavioural indicator of equine laminitis pain (Jones et al., 2007; Rietmann et al., 2004). Another behavioural indicator of laminitis pain, time spent at the back of the box (Jones et al., 2007), has also been associated with acute post-surgical limb pain (Price et al., 2003).

Conventional hoof testers are routinely used to assess solar sensitivity and to localise more sensitive sites within the foot. These tools evaluate the presence of hyperalgesia and allodynia. However, the application and interpretation of this test depends on the skill and experience of the operator and re-evaluation relies on the clinician's memory. In an attempt to adapt the principles of hoof testing

to quantitative sensory testing, Kamerling et al. (1988) produced a calibrated electronic hoof tester that allowed recording of hoof compression thresholds. The same research group also used this quantitative approach to determine the effectiveness of analgesic treatments for chronic laminitis (Owens et al., 1995; Owens et al., 1996). However, these studies presented some limitations. The hoof-tester used was still applied by hand, with potential for variability in its rate of application and in the maximum pressure applied by the hoof tester probe. Additionally, they did not provide any data on normal (sound) horses.

3.1.6 Aims and Hypotheses

In this study we aimed to further characterise the pain state associated with equine laminitis. A first aim was to evaluate a novel QST technique for the quantification of hoof compression thresholds in chronic laminitic and normal horses. A second aim was to characterise peripheral nerve morphology in the lateral digital nerve from laminitic horses and investigate injury-associated changes in protein expression in DRG neurons innervating the fore feet of laminitic horses.

We hypothesised that:

1. Chronic laminitic horses are in a state of hyperalgesia and their lowered hoof compression thresholds can be reliably measured using a novel hydraulically powered, calibrated hoof-tester.
2. The pathological processes affecting the hoof laminae during laminitis damage the sensory neurons innervating this region, giving rise to a neuropathic pain component that could explain the frequent inefficacy of standard anti-inflammatory therapy to alleviate laminitis pain.

The quantitative sensory testing, anatomical and biochemical techniques used to address these questions are detailed in Chapter 2.

3.2 Results

3.2.1 Evaluation of a novel QST technique for the assessment of mechanical hyperalgesia in chronic laminitic horses: equine laminitis is associated with mechanical hypersensitivity

Histograms, presented as Figure 3.3a and b, indicate that the threshold responses obtained from both Normal (control) and Laminitic horses can be described by a normal distribution. A not unexpected and highly statistically significant ($p<0.001$) difference was found between the mean Hoof Compression Thresholds (HCTs) of the Normal horses, 59.8 ± 4.3 kg/cm^2 (mean ± S.E.) and the mean HCTs of the Laminitic horses, 29.6 ± 3.5 kg/cm^2 (Figure 3.3c, Table 3.1). None of the fixed effects were found to be statistically significant, except for the three way interaction Group.Site.Test (*p = 0.03*), where the effect was centred on a difference of less than 3 kg/cm^2 between the first and second HCTs of Normal horses at the Lateral site.

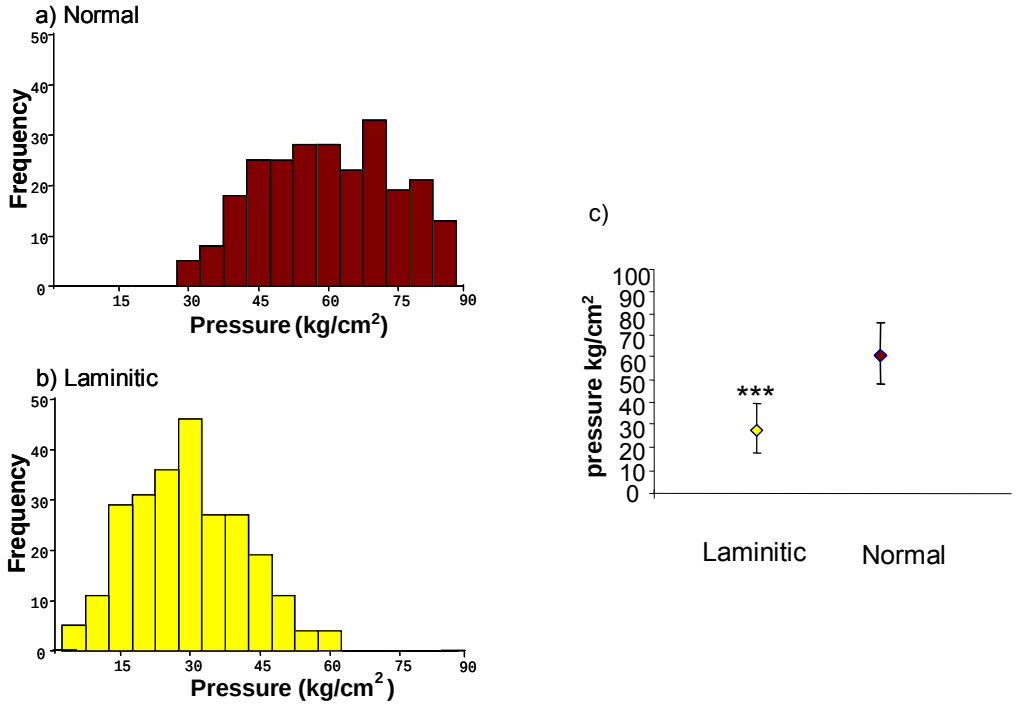

Figure 3.3: *Histograms of the frequency of occurrence of hoof compression thresholds (pressure in kg/cm^2) obtained from testing of a) Normal (n = 7) and b) Laminitic (n = 7) horses. C) Mean hoof compression thresholds (± S.E.) for laminitic (yellow) and normal (dark red) horses, expressed in kg/cm².*

Mean (± SEM) Kg/cm²	Overall	Sites			Leg	
		Toe	Medial	Lateral	Left	Right
Normal	59.8 (±4.3)	60.7 (±4.5)	57.8 (±4.5)	61.0 (±4.5)	61.7 (±4.4)	57.9 (±4.4)
Laminitic	29.6 (±3.5)	28.2 (±3.6)	28.5 (±3.6)	32.0 (±3.6)	28.7 (±3.8)	30.5 (±3.8)

Table 3.1: *Summary of QST measurements for Normal and Laminitic horses. The values are the mean Hoof Compression Test thresholds (± SEM) for Group, Sites and Legs, obtained in three Sessions with two Tests at each site, expressed in kg/cm².*

3.2.2 Evaluation of a novel QST technique for the assessment of mechanical hyperalgesia in chronic laminitic horses: Variance components analysis

Variance components, estimated separately for the HCTs of both Normal and Laminitic horses, are summarised in Table 3.2.

In both groups variance components associated with "Session", "Leg" and "Site" were consistently small. Subsequent refitting of the model without these terms showed that the properties of the model were not affected, showing that these models are consistent with the fixed effects model described earlier. However, these terms were statistically significant when they were estimated in interaction with the term "Animal" i.e. individual horses showed significant variability associated with these components.

For both Normal and Laminitic horses, the residual variance, estimated from the pairs of observations ("Tests" 1 and 2), was the largest of the variance components. This residual variance was significantly greater in the Normal group than in the Laminitic group ($p<0.01$ by *z*-test). No statistically significant differences were found between Normal and Laminitic horses for the other variance components.

In Normal horses the interaction providing the largest contribution to the total variance was that of "Animal by Session", whereas for Laminitic horses it was that of "Animal by Leg" (Table 3.2).

	Normal Horses		Laminitic Horses	
Random term	Component	S.E.	Component	S.E.
"Animal"	19.4 (19.1)	29.5 (29.6)	39.8 (37.8)	35.1 (35.0)
"Session"	0.0 *(-)*	- *(-)*	0.9 *(-)*	4.5 *(-)*
"Leg"	1.3 *(-)*	10.8 *(-)*	0.0 *(-)*	- *(-)*
"Site"	0.0 *(-)*	- *(-)*	3.0 *(-)*	4.6 *(-)*
"Animal": "Session"	44.6 *(*38.7*)*	23.6 *(*23.4*)*	8.3 (10.5)	7.0 (6.9)
"Animal" : "Leg"	9.9 (10.3)	11.7 (13.6)	26.0 (26.1)	16.7 (16.4)
"Session" : "Leg"	9.1 *(-)*	11.2 *(-)*	1.7 *(-)*	3.3 *(-)*
"Animal" : "Site"	12.6 (12.9)	8.2 (8.2)	1.5 (6.5)	5.2 (5.0)
"Session" : "Site"	1.0 *(-)*	3.2 *(-)*	0.1 *(-)*	2.3 *(-)*
"Leg" : "Site"	0.0 *(-)*	- *(-)*	0.0 *(-)*	- *(-)*
"Animal" : "Session" : "Leg"	10.0 (22.0)	11.4 (15.1)	0.4 (0.5)	4.8 (5.4)
"Animal" : "Session" : "Site"	0.0 *(-)*	- *(-)*	4.5 *(-)*	6.3 *(-)*
"Animal" : "Leg" : "Site"	0.0 *(-)*	- *(-)*	2.7 *(-)*	5.3 *(-)*
"Session" : "Leg" : "Site"	0.0 *(-)*	- *(-)*	0.0 *(-)*	- *(-)*
"Animal" : "Session" : "Leg" : "Site"	0.0 (0.0)	- *(-)*	0.0 (5.1)	- (7.6)
"Residual variance"	102.6 (103.0)	10.6 (10.6)	65.1 (65.2)	7.5 (8.3)

Table 3.2: *Estimated Variance Components and their Standard Errors for HCTs of Normal and Laminitic Horses. Values in brackets were obtained after re-fitting of the REML (restricted maximum likelihood) model. Non-statistically significant random effects are presented as either – or (-).*

3.2.3 Evaluation of a novel QST technique for the assessment of mechanical hyperalgesia in chronic laminitic horses: Statistical test evaluation: variability between animals

Further statistical analysis was carried out by calculating the intra-class correlation coefficients (ICCs) i.e. the ratio of the population variability to the total variability in the data. The ICCs were 0.50 (Normal group) and 0.57 (Laminitic group). Thus, for both groups, around half of the observed variability was associated with between-Animal variability.

The statistical sensitivity, specificity, and area under the Receiver Operating Characteristic (ROC) curves were estimated and they were 0.89; 0.86; and 0.94 respectively.

The values of the mean and variance components for each group of horses were used to determine the benefits of averaging the pairs of tests (Tests 1 and 2); this showed that the ICCs were 0.67 (Normal group) and 0.73 (Laminitic group), with an estimated sensitivity of 0.91, a specificity of 0.89 and an area under the ROC curve of 0.96.

3.2.4 Evaluation of a novel QST technique for the assessment of mechanical hyperalgesia in chronic laminitic horses: Statistical test evaluation: within Animals and between Sessions

To assess the repeatability of testing between Sessions, ICCs were determined by estimating the ratio of variability associated with "Session" to the total variability, while excluding variance components associated with between "Animal" variability. The estimated ICCs were 0.32 (Normal group) and 0.14 (Laminitic group). This indicates that, for both groups, when focusing on a single animal, little of the variability was between "Sessions".

Since most of the variability is between individual Tests, total variability could be reduced by averaging the two HCTs for each Site; the ICCs would then be 0.45 (Normal group) and 0.20 (Laminitic group).

The estimated variance components were also used to model a linear increase in the HCTs of Laminitic horses over three Sessions, until they become typical of those of Normal animals, as would occur in clinical situations in response to

effective treatment and healing. This hypothetical model was used to estimate the numbers of horses and/or test repeats (replicates) required to detect an increase (using a two-sided t-test) in the mean HCT of Laminitic horses. The increase in HCTs between sessions was modeled and quantified as the percentage reduction in the initial difference between the mean HCTs of Normal and Laminitic horses; this difference disappears as laminitics reach normality. Assuming 80% power and 95% confidence for the statistical test, examples of the numbers of horses required are shown in Table 3.3 and it can be seen that if 5 HCTs were obtained from a laminitic foot (from a randomly selected Site) in each Session for a group of nine (9) horses, there would be an 80% chance of detecting a change in mean HCT of 40% towards the mean HCT of Normal horses.

Percentage change, towards normal values, in the HCTs of a group of Laminitic horses.	**Number of tests**				
	2	3	4	5	6
	Number of horses required				
10%	40	36	34	32	32
20%	21	19	18	17	16
40%	11	10	10	9	9
80%	6	6	6	5	5

Table 3.3: *Power calculations. The table shows the number of repeated tests and number of horses in each group required to detect hypothetical improvements of 10, 20, 40, and 80% in the HCTs of Laminitic horses towards "Normal" values. A 100% improvement constitutes return to normality. Estimates were obtained from the results by modelling and a power of 80% with a confidence of 95% was assumed for the statistical testing. The table illustrates that, to detect an improvement of 40%, a group of at least 9 horses would be required and that each hoof would need to be tested at least 5 times before and after treatment.*

3.2.5 Equine laminitis is associated with morphological abnormalities in both myelinated and unmyelinated peripheral nerve fibers innervating the hoof

Lateral digital nerves dissected at the level of the proximal sesamoid bone were examined from both normal and laminitic horses. A mean of 11.65% (range 8.75–14.35) of the total fascicle area from each nerve section was analysed. EM analysis of three randomly selected fascicles per lateral digital nerve revealed morphological differences in both the myelinated and unmyelinated fibres in laminitic compared to non-laminitic horses (Table 3.4).

The most obvious quantitative feature appeared to be a significant reduction in the number of both unmyelinated (−43.2%) and myelinated fibres (−34.6%) per unit area in laminitic compared to control horses ($p = 0.016$). In order to eliminate the possibility that the reduced fibre density was an artefact due to nerve oedema, morphometric analyses were carried out to measure the percentage area of nerve sections occupied by fascicles and total nerve area in normal compared to laminitic horses. No significant differences in the mean percentages were identified ($t_4 = -0.91$, $p = 0.414$) between normals 37% (95% CI: 35.7–38.1) and laminitics 41% (39.5–42.0). No significant differences in total nerve area were identified between normal and laminitic horses ($t_4 = -0.43$, $p = 0.692$).

Further abnormalities were a significant decrease in the number of unmyelinated nerve fibres per Remak bundle ($F_{1,7} = 20.7$, $p = 0.003$) together with an increase in the percentage of solitary unmyelinated fibres in laminitics compared to normal horses ($\chi^2_1 = 35.7$, $p<0.001$, Figure 3.4 and Table 3.4b).

The percentage of morphologically damaged myelinated fibres was significantly higher in laminitic horses when compared to normal horses ($\chi^2_1 = 31.5$, $p<0.001$, Figure 3.4 and Table 3.4a). Alterations in the shape of surviving axons and disruption of the myelin sheath, with accumulation of lipid droplets and myelin debris, were the main abnormalities observed.

Finally, the proportion of myelinated fibres with continuous Schwann cell cytoplasm was significantly higher in the laminitic horses ($\chi^2 = 338.4$,

$p < 0.001$). No significant differences in myelin thickness or *G*-ratios were identified in laminitic compared to normal horses ($F_{1,7} < 0.5$, $p > 0.311$).

a) Myelinated fibres

Nerve fibre characteristics	Normal Horses n=4	Laminitic Horses n=5
Mean no. of fibres per 100 μm^2	0.52 (± 0.0)	0.34 (± 0.0) *
Mean percentage of damaged A fibres	16.40 (± 2.8)	30.08 (± 5.7) ***
Mean percentage of A fibres with continuous (>40%) Schwann cell cytoplasm	17.45 (± 1.6)	72.46 (± 5.9) ***
Mean axon diameter (μm)	5.38 (± 0.1)	5.08 (± 0.1)
Mean thickness of myelin sheath (μm)	1.06 (± 0.0)	1.09 (± 0.0)

b) Unmyelinated fibres

Nerve fibre characteristics	Normal Horses n=4	Laminitic Horses n=5
Mean no. of fibres per 100μm^2	5.77 (± 0.5)	3.28 (± 0.3) *
Mean no. of fibres per Remak bundle	2.75 (± 0.1)	2.09 (± 0.0) **
Mean percentage of solitary unmyelinated fibres	30.14 (± 2.3)	38.06 (± 5.2) ***
Mean axon diameter (μm)	1.36 (± 0.0)	1.28 (± 0.0)

Table 3.4: *Summary of myelinated and unmyelinated nerve fibre characteristics in normal and laminitic horses. Statistical significance is indicated by asterisks (* P value of <0.05, ** P<0.01, *** P<0.001; Linear mixed effects models, Mann Whitney test). Values are expressed as mean ±SEM.*

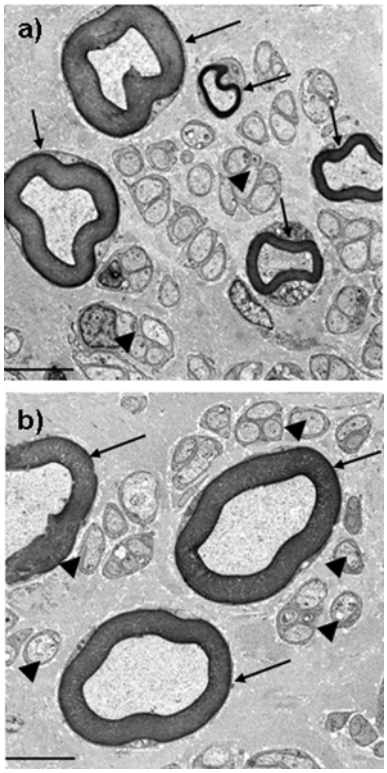

Figure 3.4: *Reduced myelinated and unmyelinated fiber density associated with laminitis. (a) Electron microscopy images of digital nerve from a normal horse. Arrows indicate intact, normal myelinated fibers. Arrowheads indicate clustered unmyelinated fibers in Remak bundles.(b) Electron microscopic images of digital nerve from a laminitic horse displaying reduced myelinated fiber density (arrows), lower numbers of C-fibers per Remak bundle, as well as increased numbers of solitary fibers (arrowheads) and increased collagen-filled space, compared to normal horse. Scale bars, 5 µm.*

3.2.6 The neuronal injury marker ATF3 is selectively expressed in sensory neurons innervating the forelimb in laminitic horses

Using immunohistochemical analysis of the DRG cell population, we assessed the presence of ATF-3 in comparison with the expression of NF-200 and either IB4 or peripherin. ATF-3 expression was significantly increased in NF-200-positive C8 DRG cells from laminitic horses (n = 3), where 67% (15 sections, 304 cells, CI 58.8–69.9) of NF-200-positive DRG cells co-expressed ATF-3, while only 10% (15 sections, 345 cells, CI 6.9–13.5) of NF-200-positive DRG cells in control horses (n = 3) co-localised ATF-3 ($\chi^2_1 = 208$; $p < 0.001$) (Figure 3.5). There was a significantly increased expression of ATF-3 in IB4-positive C8 DRG cells in laminitic horses (n = 3), where 54% (five sections, 53 cells, CI

41.5–67.3) of IB4-positive C8 DRG cells co-localised ATF-3 compared with 9% (five sections, 56 cells, CI 3.9–19.3) in control horses (n = 3) (χ^2_1 = 24.5; p < 0.001). There was also a significantly increased proportion of peripherin-positive DRG cells that were positive for ATF-3 in C8 DRG cells from laminitic horses, (n = 3), where 57% (nine sections, 115 cells, CI 47.6–65.4) of peripherin-positive cells co-expressed ATF-3 compared with 21% (nine sections, 127 cells, CI 13.8–29.4) showing double-labelling in control horses (n = 3) (χ^2_1 = 30.9; p = 0.001; Figure 3.5). Accordingly, immunoblot analysis revealed a significant increase (p < 0.05) in ATF-3 expression (expressed as mean percentage of GAPDH expression) in C8 DRG (38.9% (28.7–49.1)) in comparison to low levels in the control C4 DRG (4.6% (0.5–9.7)) (Figure 3.5 f). The numbers of cells expressing NF-200, IB4 or peripherin were unaltered in laminitic DRG compared to normal horses (696 compared to 575 NF-200-IR cells, n = 32 sections, 115 compared to 127 peripherin-IR cells, n = nine sections, 56 compared to 53 IB4-positive cells, n = five sections, in normal compared to laminitic horses, respectively).

3.2.7 Laminitis is associated with a distinctive pattern of expression of Neuropeptide Y (NPY) in sensory neurons

A significant increase in NPY immuno-reactivity was observed in the C8 DRG of laminitic horses, where 77% (17 sections, 271 cells, CI 72.0–82.3) of NF-200-positive cells showed NPY-IR co-localisation, compared to only 10% (17 sections, 351 cells, CI 15.7–24.7) in control horses (χ^2_1 = 193; p < 0:001; Figure 3.5).

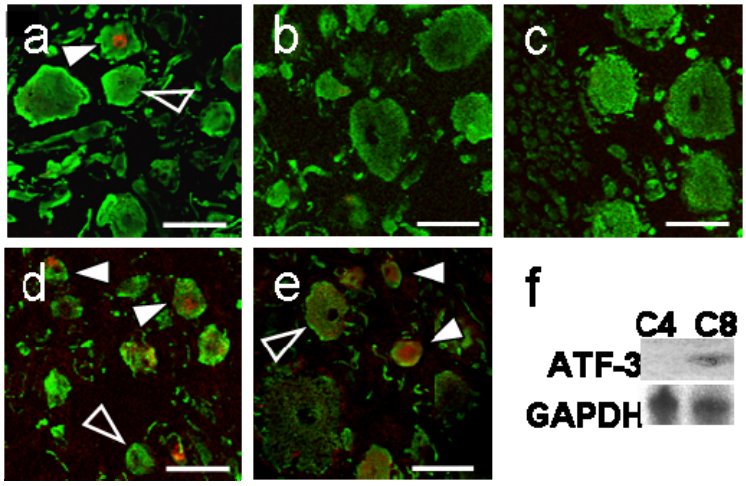

Figure 3.5: (a–e) *Immunohistochemical co-localisation of DRG neuronal subtype markers (NF-200 and peripherin, green) with neuronal injury marker, ATF-3 or neuropeptide Y (NPY) (red) in C8 DRG (which receives forelimb innervation) of laminitic (a,d,e), or control horses (b) and co-localisation of NF-200 (green) with ATF-3 (red) in C4 DRG (not associated with forelimb innervation) from the same horse (c). (a) In laminitic horses, there was an increased expression of ATF-3 (red) in NF-200-positive DRG cells (green) compared to C8 DRG control (non-laminitic) horse (b) and C4 DRG from laminitic horse (c). Laminitic horses show expression of ATF-3 (red) in peripherin-positive (green) in DRG cells (d), while control horses do not (data not shown). Additionally, there was increased co-localisation of NPY (red) and NF-200 (green) in C8 DRG cells of laminitic horses (e) compared to control horses, where there was normally only sparse NPY expression (data not shown). Scale bars, 100 lm. White arrows show co-localised immunopositive cells. Open arrows show cell marker (NF-200 or peripherin)-positive cells lacking co-localisation. (f) Typical immunoblots of whole DRG lysates of laminitic horses (n = 3) show clear ATF-3 expression in C8 but not C4 DRG. Levels of the housekeeping enzyme, GAPDH (lower blots), were unchanged.*

3.2.8 Laminitis is associated with increased expression of the TRPM8 receptor

Using immunohistochemical analysis of the DRG cell population, we assessed the presence of TRPM8 in comparison with the expression of NF-200 and peripherin. The proportions of peripherin-positive neurons found to express TRPM8 were significantly higher in C8 DRG from laminitic horses compared to C8 from normal horses and C4 from laminitic horses ($p < 0.0001$). Tukey's *post hoc* tests indicated that there was no statistical difference between the number of double-labelled neurons in C8 DRG from normal horses and C4 DRG from laminitic horses. 7.60% ± 0.46 of peripherin-positive cells in the C8 DRG from

laminitic horses co-expressed the TRPM8 receptor, compared to 3.95% ± 0.33 in C8 DRG from normal horses and 3.20% ± 0.41 in C4 from laminitic horses (Figure 3.6a-c). The proportions of NF-200-positive neurons found to express TRPM8 were significantly higher in C8 DRG from laminitic horses compared to C8 from normal horses and C4 from laminitic horses ($p < 0.0001$). Tukey's *post hoc* tests indicated that there was no statistical difference between the number of double-labelled neurons in C8 DRG from normal horses and C4 DRG from laminitic horses. 5.64% ± 0.57 (mean ± SE) of NF-200-positive neurons from the C8 DRG of laminitic horses were immuno-positive for the TRPM8 receptor, compared to 2.38% ± 0.1 in C8 DRG from normal horses and 2.23% ± 0.22 in C4 DRG from laminitic horses (Figure 3.6d-f).

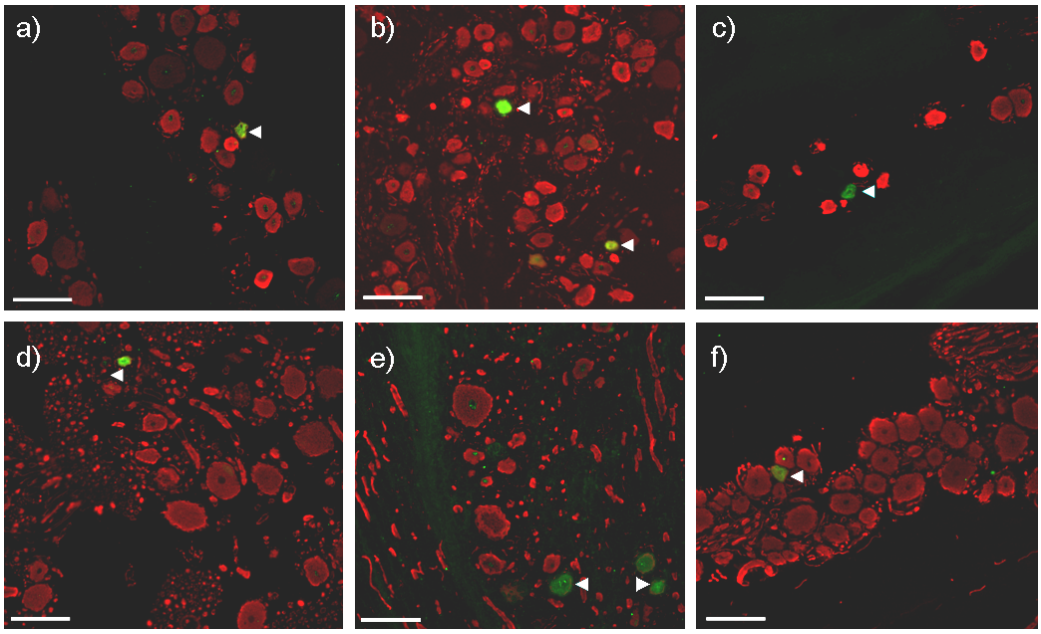

Figure 3.6: *Immunohistochemical co-localisation of DRG neuronal subtype markers (NF-200 and peripherin, red) the TRPM8 receptor (green) in C8 DRG (which receives forelimb innervation) of control horses (a, d) and laminitic horses (b,e) and in C4 DRG (not associated with forelimb innervation) from the same laminitic horses (c,f). Laminitic horses (b) show an increased expression of TRPM8 (green) in peripherin-positive DRG cells (red) compared to C8 DRG control (non-laminitic) horse (a) and C4 DRG from laminitic horse (c). Laminitic horses also show increased expression of TRPM8 (green) in NF-200-positive DRG cells (red) (e), compared to C8 from control horses (d) and C4 DRG from the same laminitic horses (f). Scale bars, 100 μm. White arrows indicate examples of co-localised immunopositive cells.*

3.3 Discussion

3.3.1 QST assessment of laminitis-associated pain

Equine laminitis is a relatively frequent disease of horses and ponies which is generally perceived by veterinarians to be associated with a variable degree of pain according to the severity of its presentation (Price et al., 2002). Clinical assessment of pain associated with laminitis continues to be largely subjective and relies heavily upon the expertise and skills of the clinician. Although a number of subjective pain scales (such as the OBEL score) have been developed in an attempt to standardise the assessment of pain in laminitic horses, these methods present a number of limitations, mainly regarding their reproducibility when used by different observers on the same animal and the role of experience in their performance (Vinuela-Fernandez et al., 2010). Therefore, there is a need for objective, quantitative methods for the assessment of laminitis pain. Quantification of pain behaviours has been shown to provide a reliable method of assessing pain in laminitic horses. We have previously shown that behaviours such as foot lifting or time spent at the back of the box are significantly increased in laminitic horses compared to normal horses (Jones et al., 2007). However, a disadvantage of behavioural quantification in a clinical setting is that it requires video recording of the animals for long periods of time and data processing and analysis is time consuming.

Quantitative sensory testing protocols are routinely used in human medicine to assess sensory nerve function and several QST methods have been developed in recent years for pain assessment in animals. In human patients, QST allows for the evaluation of qualitative aspects of sensory perception that can be communicated through verbal self-report but this is not possible in animals. QST tests developed for animals use behavioural responses (e.g. a withdrawal response) as an end-point to measure nociceptive thresholds.

In this study, we have confirmed and extended the measurements of noxious mechanical thresholds on the forefeet of lame horses, presented by Kamerling et al. (1988). They reported Hoof Compression Thresholds (HCTs) and the number of loci which were pain sensitive for 6 horses with lameness from various foot

problems (they did not test 'normal' horses). The range of HCTs found when converted to kg/cm^2 was from 18.9 ± 3.4 to 46.4 ± 12.0 with a mean ± SEM of 24.9 ± 3.2 kg/cm^2, which is consistent with the mean reported here for laminitic horses (29.6 ± 3.5 kg/cm^2). The highest HCTs in their range are quite close to the mean reported here for normal horses (59.8 ± 4.3 kg/cm^2). Kamerling et al. (1988) found decreases in HCTs (hyperalgesia) after an undefined number of repeated tests. Hyperalgesia was also found as a result of repeated hoof testing when using the same calibrated hoof tester to measure the effectiveness of detomidine, ketoprofen and phenylbutazone as analgesics for chronic hoof pain (Owens et al., 1995; Owens et al., 1996). Owens et al. (1995; 1996) measured proportional changes in the HCTs of laminitic horses following treatment, and expressed the effectiveness of treatments as the percentage increase in HCTs with reference to the baseline. They did not present absolute HCT values for either normal or laminitic horses. Kamerling's et al. (1988) calibrated hoof tester was applied manually over a period of about 2 seconds and the maximum pressure applied was often determined by the grip strength of the examiner, which is clearly not ideal. Results from the present study suggest that increasing probe pressure at the slower rate of about 10 kg/s/cm^2 and prevention of overstimulation by automatic control of the maximum pressure avoids excessive stimulation and prevents development of hyperalgesia. In the horses tested, no damage was detected by clinical examination for lameness and no evidence was found, from decrease in the HCTs over time, that hyperalgesia developed as a result of repeated testing.

The HCT thresholds for the group of chronically laminitic horses were generally less than 50% of those obtained for 'normal' control horses and this can be related to a widely accepted view of the severity of foot pain suffered by horses with chronic laminitis.

In order for a new QST method to be established for routine assessment its reliability needs to be evaluated. This study approached this by analyzing the sources of variation and calculating intra-correlations coefficients.

In both normal and laminitic horses, the "residual variance" component, which reflects variability between the first and the second test within a session in

individual horses plus any unaccounted for error, was the largest element of the variance components, and it was significantly greater in normal than laminitic horses (Table 3.2). This higher variance in normal horses compared to laminitics may be attributed to the lower HCTs of laminitic horses, which may reduce opportunities for interfering variables to produce measurement errors and lower the likelihood of coincident voluntary movements of the horse which would obscure detection of the threshold. No published quantitative evidence has been found with which to compare these differences in the variability of HCTs between normal and laminitic horses as most previous studies do not provide data on normal animals.

Variability between different horses was a large source of variability in the data, particularly for the group of laminitic horses. This is likely to be due to these horses being at different stages of disease progression or to differences in their responses to therapy.

In normal horses, one of the largest contributions to variability was a result of "Animal by Session" interaction (i.e. the variability in HCTs of a particular horse from one session to another). This was confirmed by calculation of intra-class correlation coefficients which showed that, in normal horses, about one third of the total variability is associated with between session variability. Such high variability in normal horses over time is a limiting factor in further studies using HCTs for the measurement of treatment effects or disease progression but such reference values are required as it is unlikely that control data from the laminitic horses prior to the development of the disease would normally be available.

The benefits of averaging of various sampling factors were modelled and averaging of HCTs from different sites in the same 'leg' (foot) seems likely to be a useful strategy for improving the effectiveness of the test. The magnitude of the variance associated with an "Animal by Leg" interaction in laminitic horses, might correspond to differences in the sensitivity of the left versus right foot of individual animals. It is a common clinical finding that one of the feet is generally more severely affected than the other and this was also reported by Kamerling et al. (1988). Thus averaging of HCTs from both fore-feet together, would not be beneficial, for assessment of changes in the severity of laminitis.

In neither normal nor laminitic horses was there any strong evidence of differences in the statistical properties of HCTs measured at the three different sites. A single site, the Toe site (half-way between the apex of the frog and the white line), would be easier to use as a consistent anatomical location for testing.

Differences between the first and second HCT in normal horses at the lateral site were statistically significant, although relatively small (<3 kg/cm^2), and it was not dependent on the right or left foot (leg). We believe this unexpected finding should be taken cautiously and it needs to be confirmed before further investigation of or speculation on its origin is pursued.

Owens et al. (1995; 1996) concluded that, because of underlying variability, relatively large and consistent changes in HCTs are required to show statistically significant improvements in response to treatment. The results presented here are consistent with this and provide further insight into the sources of the variability. No direct evidence was obtained here to support their proposal that quantitative measurement of HCTs provides a better means for detecting analgesic effects than subjective assessment of changes in lameness obtained using the Obel scoring method. More studies, in which both methods of assessment are used on the same horses at the same time, are required to test this proposal.

In conclusion, this part of the study shows that HCTs can be safely and reliably measured, under experimental conditions, using this hoof-tester. The variability found in the HCTs indicates that treatments need to produce at least a 40% improvement in order to be detected in a group of nine laminitic horses tested at least five times. Clinical application of the method, which requires measurement of meaningful changes in the HCTs of individual laminitic horses, may be achieved after successful investigation and implementation of one or more of the following potential improvements:

- Decreasing the variability between measurements on the same foot of an individual horse by rigorous attention (including training) to: the horse's 'mental state', its behavioural responses to hoof-testing, precise location of the instrument, and improvements in the ability of the operator to reliably detect the HCT threshold (end-point) for a particular horse.

- Finding well-defined and easily applied criteria for the rejection of HCT threshold measurements affected by co-incident variables such as environmental disturbances or failure of the horse to cooperate.
- Accommodation of variability by averaging out some of the errors. Most variability occurs at the test stratum and we suggest that the averaging of five (5) well-controlled HCTs, performed at one site (Toe) on the sole of each foot, might be safely achieved without cumulative deleterious effects.
- Because of the complexity and high cost of this hoof tester, development and testing of simpler and cheaper calibrated hoof-testers, would help clinical exploitation of HCTs. We suggest that, in order to help ensure the welfare of horses being tested, specifications for and use of calibrated hoof testers should be consistent with the protocols and safe 'limits' described here.

3.3.2 Abnormal hoof sensory nerve morphology in laminitic horses is consistent with that reported in damaged peripheral nerves in neuropathic pain states

Damage to sensory nerves has been associated with spontaneous pain and increased nociceptive sensitivity in a variety of clinical and experimental studies. In this study, we have quantified a reduction in mechanical nociceptive thresholds associated with equine laminitis (compared to normal horses) which is indicative of a hypersensitive sensory state.

Changes in sensory nerve fibres innervating the hoof could potentially contribute to the aetiology of the chronic laminitic pain state. To understand the mechanisms underlying laminitis pain and the incomplete response to anti-inflammatory analgesics, it is important to establish whether axonopathic changes may contribute. In laminitic horses, EM analysis identified marked decreases in myelinated and unmyelinated fibre numbers per unit area of digital nerve. This is unlikely to be due to nerve oedema as there were no significant differences between nerve section areas and fascicle areas in normal and laminitic horses. The marked increase in the number of solitary, unmyelinated

fibres may reflect demyelinated A-fibres or an absence of guiding pathways for regenerating C-fibres (Bester et al., 1998). The morphological changes observed are consistent with those in laboratory neuropathic pain models, such as chronic constriction injury (Basbaum et al., 1991; Gabay and Tal, 2004; Gautron et al., 1990; Micu et al., 2006), crush injury (Lozeron et al., 2004), photochemically-induced ischemia (Yu et al., 2000) and diabetic neuropathy (Elias et al., 1998; Kalichman et al., 1998; Llewelyn et al., 1991; Sima et al., 1988), thereby supporting our hypothesis that peripheral nerve damage may contribute to laminitis pain.

Functional changes in the injured peripheral nerve have also been described in neuropathic pain models.

The loss of large fibres in nerves from laminitic horses is important as part of the behavioural changes in neuropathic pain states may result from the loss of spinal inhibitory controls exerted indirectly by these afferents (Basbaum et al., 1991). On the other hand, damage to both A and C-fibres appears to be necessary for the establishment of hyperalgesia and allodynia (Gabay and Tal, 2004; Yu et al., 2000). Electrophysiological studies further suggest that ectopic discharges in both spared C- and A-fibres may be important in maintaining neuropathic pain (Ali et al., 1999; Gabay and Tal, 2004; Kajander and Bennett, 1992).

3.3.3 Sensory neurons of the forelimb in laminitic horses show phenotypic changes associated with peripheral nerve injury

Following peripheral nerve damage, phenotypic changes occur in primary sensory neurons that may contribute to mediating central sensitisation (Hokfelt et al., 1994; Tsujino et al., 2000). We assessed whether key neurochemical changes in sensory neurons of laminitic horses are similar to those in rodent neuropathic pain models. The numbers of DRG cells expressing the anatomical markers NF-200, peripherin or IB4-binding sites were unaltered. Following nerve crush injury, peripherin increases transiently in large DRG cells (Wong and Oblinger, 1990). However, that model is associated with sensory loss (Bester et al., 1998) rather than the hypersensitivity seen here, as in other neuropathic and inflammatory pain states, where indeed peripherin expression is not upregulated (Facer et al., 2007; Renton et al., 2003; Rodriguez et al., 2006).

Neuronal expression of ATF-3, a member of the ATF/CREB family of transcription factors which is normally minimal, is upregulated following peripheral nerve injury and so can act as a marker of frank nerve damage (Tsujino et al., 2000). The clear expression of ATF-3 in NF-200, peripherin or IB4-positive sensory neurons of laminitic horses compared to normal horses indicates neuronal damage to both A and C-fibres matching our observations of abnormal nerve morphology. These findings suggest that primary afferent injury associated with laminitis arises locally from the damage caused by hoof pathology, rather than from systemic disease, since ATF-3 expression is low in neurons from DRG not innervating the forelimb. ATF-3 has been demonstrated to be induced by a number of stress signals in a wide range of tissues (Chen et al., 1996a; Yin et al., 1997). Ischemia and ischemia/reperfusion are established causes of ATF-3 expression (Hai et al., 1999), so the ischemia- reperfusion injury of the digit thought to underlie acute laminitis (Hood, 1999) may also be involved in neuronal damage.

Immediate early-genes (IEGs) play a role in adaptive plasticity and long-term changes in the nervous system. Several IEGs, with which ATF-3 shares many characteristics (Wolfgang et al., 1997), have been shown to characterise the expression profile after peripheral nerve injury (Herdegen and Zimmermann, 1995). ATF-3 represses transduction as a homodimer and activates transcription as a heterodimer with Jun proteins (Chen et al., 1994; Hai and Curran, 1991). ATF-3 is closely related to c-Jun, which is one of the best characterised early IEGs and has been shown to be induced in neurons following axotomy and is thought to be related to axonal regeneration (Chen et al., 1994; Jenkins and Hunt, 1991; Leah et al., 1991). Although the function of ATF-3 has not been elucidated in detail, it has been suggested that the pattern of ATF-3 and c-Jun co-expression post-injury might be involved in determining the fate of DRG neurons following axotomy (Tsujino et al., 2000).

In addition to increased expression of ATF-3 we also found upregulated expression of neuropeptide Y (NPY) in large NF-200-positive DRG cells from laminitic horses, paralleling observations in other neuropathic pain models (Kashiba et al., 1994; Ma and Bisby, 1998; Munglani et al., 1995; Nahin et al.,

1994; Noguchi et al., 1993; Wakisaka et al., 1991; Wallace et al., 2003). NPY has been associated with cross excitation between A and C fibres, through correlation of increased NPY in large DRG neurons and the increase in NPY receptor Y2 in small cells following nerve injury (Noguchi et al., 1993; Wakisaka et al., 1991; Wakisaka et al., 1992; Zhang et al., 1997).

In conclusion, the novel findings reported here suggest that pathological changes occurring during laminitis bring about a chronic pain state with a neuropathic component.

Although the mechanisms underlying the pathogenesis of laminitis remain to be fully elucidated, it is apparent that the early stages of laminitis are associated with vasoconstriction of the digital microvasculature (Peroni et al., 2006) and inflammation (Belknap et al., 2007). Indeed, such pathological events can result in nerve damage (Moalem and Tracey, 2006; Yu et al., 2000; Zimmermann, 2001) and may thereby play a role in the pathobiology of laminitis pain through the transition from acute inflammatory pain to a chronic syndrome with a neuropathic pain component. Additionally, enzymatic events characteristic of the developmental phase of laminitis, such matrix-metalloproteinases (particularly MMP-2 and MMP-9) might also have a role in the development of neuropathic pain component (Kawasaki et al., 2008; Kyaw-Tanner and Pollitt, 2004; Loftus et al., 2006).

Neuropathic changes may be, at least partially, responsible for the limited efficacy of currently used anti-inflammatory therapy in the treatment of laminitis pain. The administration of anti-neuropathic agents may therefore achieve better pain management and improved quality of life in horses suffering from refractory laminitis.

3.3.4 Equine laminitis is associated with increased expression of TRPM8 in sensory neurons innervating the forelimbs

Immunohistochemical analysis of neuronal bodies from the DRG innervating the forelimb (C8) showed an increased expression of the TRPM8 channel, compared with those from a cervical DRG not involved in forelimb innervation (C4) and also with the C8 DRG from non-laminitic horses. The increase in TRPM8 expression was observed in both peripherin and NF200-positive cells.

TRPM8 expression has been shown to increase in peripherin-positive DRG neurons ipsilateral to CCI nerve injury in rats and also to be newly expressed in small myelinated (NF-200-positive), presumed-Aδ fibre cells (Frederick et al., 2007; Proudfoot et al., 2006). However, no alterations in TRPM8 expression were reported in the SNL model of nerve injury, nor in a model of inflammation, (Katsura et al., 2006; Obata et al., 2005).

This finding provides, to the author's knowledge, the first evidence of TRPM8 upregulation in a naturally occurring painful disease in a domestic animal species. Moreover, in the context of previous studies that identified a role for TRPM8 as an analgesic system that can be successfully recruited in chronic pain models, (Dhaka et al., 2007; Proudfoot et al., 2006), the findings reported here support the use of TRPM8 agonists for the treatment of equine laminitis pain.

Chapter 4: 5-HT1B RECEPTOR MODULATION OF TRPM8 CHANNEL ACTIVITY

4.1 Introduction

4.1.1 Serotonin and serotonin receptors

Serotonin (5-hydroxytryptamine; 5-HT) is a monoamine neurotransmitter that is primarily found in the gastrointestinal tract, platelets and the nervous system. About 80% of the human body's serotonin is found in enterochromaffin cells in the intestinal wall, where it regulates intestinal movements. Serotonin is also involved, for example, in the modulation of cardiovascular function, ejaculatory latency, and bladder control (Berger et al., 2009). In the CNS, serotonin is almost exclusively produced in neurons from the raphe nuclei, located in the midline of the brainstem, that send ascending projections to higher brain structures and descending projections to the spinal cord. Serotonin receptors are differentially expressed in all areas of the CNS, and thus serotonin is involved in the modulation of practically all its functions (Berger et al., 2009). Importantly, individual neurons might express multiple serotonin receptors, sometimes with opposing functions (Araneda and Andrade, 1991). Dysregulation of the serotonergic system has been implicated in the pathogenesis of many psychiatric and neurological disorders (Roth, 1994).

In neurons, serotonin is synthesized from tryptophan by conversion to 5-hydroxytryptophan by the enzyme tryptophan hydroxylase, followed by decarboxylation to 5-HT by a nonspecific amino acid decarboxylase. 5-HT is transported back into nerve terminals via a specific serotonin transporter (SERT), which effectively ends the synaptic action of 5-HT and enables its degradation by monoamine oxidase.

Major advances in the understanding of serotonin function have been made over the last two decades with the cloning of 15 serotonin receptor subtypes (Kroeze et al., 2002). 5-HT receptors have been classified on the basis of molecular similarity, pharmacology and transduction mechanisms (Hoyer et al., 1994).

There are 7 families of 5-HT receptors, classified as 5-HT_{1-7} with further subdivision of 5-HT_1 into 5-HT_{1A-B}, 5-$HT_{1D/F}$ and 5-ht_{1E}; 5-HT_2 into 5-HT_{2A-C}; 5-HT_3 into 5-HT_{3A-B} and 5-ht_5 into 5-$ht_{5A/B}$ (Hannon and Hoyer, 2008). In terms of nomenclature, uppercase represents those receptors for which functional data is available in native tissue and lowercase, e.g. 5-ht_{5A-B}, indicates that no clear functional role has been yet established. The 5-ht_{5B} receptor is not expressed in man (Hannon and Hoyer, 2008).

Like glutamate, serotonin acts through both metabotropic and ligand-gated channels. Of the 7 families of 5-HT receptor only the 5-HT_3 receptor is a ligand-gated ion channel; its activation triggers rapid depolarisation by opening a non-selective cation-permeable pore, causing influx of Na^+ and Ca^{2+} and efflux of K^+ (Hannon and Hoyer, 2008; Hoyer et al., 2002). The remainder are type A G protein-coupled receptors (GPCRs), which show a seven-transmembrane-structure (Findlay and Eliopoulos, 1990; Trumpp-Kallmeyer et al., 1992). 5-HT_1 receptors are linked to Gi/o and therefore exert an inhibitory effect on adenylate cyclase, inhibiting cAMP formation; whereas 5-HT_2 receptors preferentially couple to Gq/11 and thus promote PLC activation increasing inositol phosphates and cytosolic Ca^{2+} concentrations. 5-HT_4, 5-HT_6 and 5-HT_7 receptors preferentially couple to Gs and activate adenylate cyclase resulting in cAMP production (Hannon and Hoyer, 2008; Hoyer et al., 2002).

4.1.2 Serotonin and pain

Serotonin is involved in the modulation of nociceptive processing and pain perception at multiple levels of both the peripheral and the central nervous systems. During inflammation, the release of serotonin sensitises primary afferent neurons that carry nociceptive information to the CNS (Sommer, 2004). Serotonergic neurons in the brainstem send descending projections to the spinal cord, thus modulating incoming nociceptive inputs (Braz et al., 2009). Additionally, serotonergic neurons from the midbrain send ascending projections to limbic and cortical areas that may be involved in the affective-motivational component of pain (Li et al., 1993).

4.1.3 Serotonin receptors in nociceptive processing in spinal cord

Although a subset of 5-HT-immunopositive cells has been identified in the spinal cord of primates, ventral to the central canal (LaMotte, 1988), practically the entire serotonergic innervation of the spinal cord originates from supraspinal structures (reviewed by Millan, 2002). 5-HT-immunopositive nerve fibres, observed as immunoreactive varicosities and nerve terminals, have been identified in the spinal cord, with highest immunoreactivity in lamina I and II_o although they are also present in deeper laminae (Marlier et al., 1991; Maxwell et al., 1996; Mizukawa et al., 1986). The main descending serotonergic pathways originate from the nucleus raphe magnus (NRM), a medullary structure innervated by the periaqueductal grey (PAG) (Bowker et al., 1983; Kwiat and Basbaum, 1992; Mason, 1999). Acute and chronic noxious stimulation results in the activation of descending serotonergic pathways and increased serotonin levels in the dorsal horn of the spinal cord (Mason, 1999; Taguchi and Suzuki, 1992; Weil-Fugazza et al., 1979; Weil-Fugazza et al., 1984). Descending serotonergic pathways are involved in the modulation of spinal cord nociceptive processing, exerting either inhibitory or facilitatory influences. Spinal cord 5-HT receptors are located on primary afferent terminals, projection neurons and inhibitory and excitatory interneurons (Millan, 2002). The location and possible functional role of the most relevant 5-HT receptor subtypes found in the spinal cord is briefly summarised as follows:

5-HT_1 receptors

5-HT_1 receptors play largely an inhibitory role in nociceptive transmission in the spinal cord. Whereas the 5-HT_{1A} receptor is localised post-synaptically, mainly in the superficial dorsal horn, 5-HT_{1B}, 5-HT_{1D} and 5-HT_{1F} receptors are predominantly pre-synaptic (Chen et al., 1998; el-Yassir and Fleetwood-Walker, 1990; el-Yassir et al., 1988; Zemlan and Schwab, 1991). Spinal administration of selective 5-HT_{1A} receptor agonists has been shown to attenuate hypersensitivity and nocifensive behaviours in rodent models of inflammatory and neuropathic pain (Bardin et al., 2003; Deseure et al., 2007). Triptan drugs, which act on 5-

HT receptor subtypes 1B, 1D and 1F are widely used for the treatment of migraine pain (Johnston and Rapoport, 2010). Moreover, intrathecal sumatriptan has been shown to reduce pain hypersensitivity in mice models of persistent inflammatory and visceral pain but not in the SNI model of neuropathic pain (Nikai et al., 2008). Sumatriptan appears to act on pre-synaptic 5-HT_{1D} receptors and the lack of efficacy of this drug to alleviate neuropathic pain has been suggested to be related to the down-regulation of 5-HT_{1D} in the central terminals of primary afferents identified following nerve injury (Ahn and Basbaum, 2006; Jennings et al., 2004; Nikai et al., 2008). Effects of other 5-HT_{1B} agonists and antagonists (el-Yassir et al., 1988; Gjerstad et al., 1997; Liu et al., 2007) are also consistent with an antinociceptive role of 5-HT_{1B} receptors, although one report suggested a pro-nociceptive role (Zhang et al., 2001). Data from knock-out mice has confirmed the inhibitory role of 5-HT_1 receptors subtypes in nociception. Mice lacking 5-HT_{1A} receptors showed higher sensitivity to the hot-plate test than their wild-type counterparts, whereas 5-HT_{1B} receptor knock-out mice exhibited higher thermal and formalin sensitivity than wild-types (Kayser et al., 2007).

5-HT_2 receptors

5-HT_{2A} receptors are largely expressed pre-synaptically in the dorsal horn but also in moderate levels post-synaptically and in inhibitory neurons where their activation has been suggested to mediate antinociceptive responses (Fonseca et al., 2001; Li et al., 2000; Maeshima et al., 1998; Sasaki et al., 2003). Data from knock-out mice however appears to suggest that 5-HT_{2A} receptors are pronociceptive as 5-HT_{2A} receptor null mice exhibited a decrease in the nociceptive responses of the second phase of the formalin test (Kayser et al., 2007).

5-HT$_3$ receptors

5-HT$_3$ receptors are located in the central terminals of small primary afferents and axons from excitatory interneurons in the superficial dorsal horn where they exert pronociceptive influences (Conte et al., 2005; Zeitz et al., 2002). Intrathecal administration of the selective 5-HT$_3$ receptor antagonist ondansetron has been shown to attenuate pain hypersensitivity in models of neuropathic and inflammatory pain (Green et al., 2000; Rahman et al., 2009; Suzuki et al., 2004). Indeed, it has been suggested that descending facilitatory influences originating at NK1 receptor-positive neurons in the dorsal horn and relaying through the RVM are enhanced following nerve injury or formalin-induced inflammation, and that these act on facilitatory spinal 5-HT$_3$ receptors that increase neuronal excitability (Suzuki et al., 2002). However, some reports have suggested that the facilitatory actions of 5-HT$_3$ receptors might be exerted on GABAergic inhibitory neurons in the superficial dorsal horn, resulting in antinociception (Fukushima et al., 2009).

5-HT$_4$, 5-HT$_6$ and 5-HT$_7$ receptors

5-HT$_4$, 5-HT$_6$ and 5-HT$_7$ receptors are positively coupled to adenylate cyclase and thereby have a facilitatory effect on neuronal excitability (Hoyer et al., 2002). Antagonism of 5-HT$_4$ receptors has been reported to mediate antinociception, especially inputs from enteric viscera and in relation to formalin-induced inflammation (Doak and Sawynok, 1997; Espejo and Gil, 1998). Spinal 5-HT$_7$ receptors have also been suggested to play a pronociceptive role in formalin-induced inflammatory pain (Rocha-Gonzalez et al., 2005).

4.1.4 Peripheral serotonin receptors

Serotonin receptors have been implicated in the modulation of spinal nociceptive transmission through actions both on the central terminals of primary afferents and in spinal cord neurons, as described above. Additionally, recent studies have investigated the role of peripheral 5-HT receptors in pain processing. 5-HT is released by platelets and mast cells following tissue injury

and is thus considered an inflammatory mediator (Dray, 1995). Similarly, nerve injury following sciatic nerve transection and CCI also results in increased levels of serotonin in the injured nerve (Anden and Olsson, 1967; Vogel et al., 2003).

Intraplantar 5-HT administration results in concentration-dependent increased nociceptive behaviour (i.e. paw lifting and licking) in rats, and these effects are blocked with pre-treatment using specific 5-HT receptor antagonists (Sufka et al., 1992).

Serotonin receptors have been suggested to be present in primary afferents. A study using in situ immunohybridization in rat DRG neurons identified mRNAs for 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{3B} and 5-HT_4 receptors. However, mRNAs for 5-HT_{1A}, 5-HT_{1E}, 5-HT_{2C}, 5-HT_{5A}, 5-HT_{5B}, 5-HT_6 and 5-HT_7 receptors were not detected in these neurons (Nicholson et al., 2003). Immunohistochemical localisation of 5-HT_{2A} receptors in peripheral sensory axons of rat glabrous skin has provided anatomical evidence that 5-HT can have a direct effect on primary afferents (Carlton and Coggeshall, 1997). However, the functional role of peripheral 5-HT receptors has not yet been fully elucidated (Sommer, 2004). Different receptors appear to mediate different, sometimes even antagonistic effects. Intradermal injection of a 5-HT_{1A} receptor agonist in rats resulted in a rapid development of hyperalgesia whereas 5-HT_{1B}, 2-HT_{2C} and 5-HT_3 receptor agonists did not have that effect (Taiwo and Levine, 1992). Administration of 5-HT_2 receptor agonists can also result in nociceptive sensitisation or evoke nociceptive behaviours, particularly when co-administered with 5-HT or other inflammatory mediators (Abbott et al., 1996; Okamoto et al., 2002; Tokunaga et al., 1998). The 5-HT_{2A} receptor has been further implicated in inflammatory pain. Administration of a 5-HT_{2A} receptor antagonist reduced thermal hyperalgesia caused by CFA but did not have analgesic effects in CCI rats (Okamoto et al., 2002). Furthermore, it was found that levels of 5-HT_{2A} receptor mRNA in DRG neurons were increased following CFA, but not after axotomy or CCI (Okamoto et al., 2002). 5-HT_{2A} receptor antagonists have also been found to attenuate pain behaviours in response to the formalin test in rats (Abbott et al., 1997; Obata et al., 2000). Interestingly, 5-HT receptors can also mediate direct analgesic effects. Peripheral administration of 5-HT_1 receptor

specific agonists for the 1A, 1B, 1D, 1F subtypes resulted in decreased nocifensive behaviours in the formalin model of pain in rats and this effect was abolished by co-administration of specific 5-HT_1 receptor antagonists (Granados-Soto et al., 2010). Systemic administration of the 5-$HT_{1B/D}$ receptor agonist sumatriptan reduced thermal hyperalgesia in an inflammatory pain model (carrageenan) in mice but not in a sciatic nerve ligation model of neuropathic pain in rats through an apparently 5-HT_{1D} receptor-mediated mechanism (Bingham et al., 2001), although efficacy was reported in a model of trigeminal neuropathic pain (Kayser et al., 2002). Interestingly, it has been recently suggested that the analgesic effect of *Heterotheca inuloides*, a plant used in traditional Mexican medicine for its anti-inflammatory properties is mediated by 5-HT_1 receptors (Rocha-Gonzalez et al., 2010).

4.1.5 5-HT_{1B} receptors

Distribution and function

5-HT_{1B} receptors are widely distributed within the nervous system. In the CNS they are highly expressed in the basal ganglia and cerebellum but are also moderately expressed in other brain areas like the hippocampus, periaqueductal gray and frontal cortex (for a review see Sari, 2004). 5-HT_{1B} receptors have been shown to play a functional role in locomotion and to be involved in psychiatric disorders such as anxiety, depression, alcoholism and aggressive behaviour (Boulenguez et al., 1995; Brunner et al., 1999; Huang et al., 2003; Kaiyala et al., 2003; Saudou et al., 1994). 5-HT_{1B} receptors are expressed both in serotonergic and non-serotonergic neurons, where they act as autoreceptors (regulating serotonin release) and pre-synaptic heteroreceptors (inhibiting neurotransmitter release), respectively (Engel et al., 1986; Maura and Raiteri, 1986; Sarhan et al., 2000). 5-HT_{1B} receptors, which are of particular interest here are expressed in a subpopulation of afferents, mainly TrkA positive, SP-negative IB4-negative Aδ and C fibres (Hou et al., 2001; Ma et al., 2001; Wotherspoon and Priestley, 2000).

The 5-HT_{1B} receptor in rat and mouse is composed of 386 amino-acids whereas its human homologue, formerly known as 5-$HT1_{D\beta}$ and recently renamed as h-5-HT_{1B}, is composed of 390 amino-acids (Sari, 2004). It appears that structural differences between rodent and human 5-HT_{1B} receptor are reflected by some differences in their pharmacological properties. For example, rodent 5-HT_{1B} receptors bind to β-adrenergic antagonists with higher affinity than the h-5-HT_{1B} receptor and this has been shown to be due to a single amino acid difference (Metcalf et al., 1992). 5-HT_{1B} receptors carry out their signal transduction mechanisms through their coupling to guanine-nucleotide binding proteins (G-proteins), especially those in the Gi/Go family. Thus their activation results in inhibition of adenylate cyclase activity, decreasing the production of cAMP from ATP (Hamblin and Metcalf, 1991; Maroteaux et al., 1992; Seuwen et al., 1988). Additionally, activation of 5-HT_{1B} receptors has been shown to activate p70 S6 kinase and the mitogen-activated protein kinase (MAP kinase) ERK-2 (Pullarkat et al., 1998). Moreover, studies on smooth muscle contraction have shown that serotonin can stimulate phospholipase D (PLD) via the 5-HT_{1B} receptor (Hinton et al., 1999).

4.1.6 TRPM8 channel and chronic pain states

As fully described in Chapter 1, activation of the TRPM8 channel has been shown to produce a reversal of the behavioural reflex sensitisation to noxious stimuli associated with chronic pain states (Proudfoot et al., 2006). Interestingly, TRPM8 channel activation did not result in modification of nociceptive reflexes in uninjured contralateral limbs or in normal animals (Proudfoot et al., 2006). This suggests that TRPM8 channel activity must be somehow modulated in chronic pain states.

TRPM8 channels are expressed by particular subsets of afferent neurons. TRPM8-expressing neurons comprise both A-fibre and C-fibre neurons also expressing TrkA, and are generally distinct from IB4-positive and SP-positive neurons. (Dhaka et al., 2007; Kobayashi et al., 2005; Nealen et al., 2003; Story et al., 2003).

4.1.7 Aim and Hypotheses

The programme of work detailed below was carried out to investigate modulatory effects at the TRPM8 channel that could potentially influence its observed analgesic effect in chronic pain states. Since, in broad terms, the profile of afferents expressing TRPM8 channels correlates with the profile expressing 5-HT_{1B} receptors and serotonin plays a role in both inflammatory and neuropathic pain we aimed to explore the possibility that 5-HT_{1B} receptors could potentially influence TRPM8 channel function.

We hypothesised that activation of 5-HT_{1B} receptors would result in a facilitatory effect on chemically-mediated TRPM8 channel activity and that this effect is mediated through PLD1, possibly leading to an increased production of PIP_2. We also hypothesised that co-administration of a 5-HT_{1B} receptor agonist and a TRPM8 channel agonist would result in increased analgesia in a rodent model of neuropathic pain.

4.2 Results

4.2.1 Characterisation of responses to icilin in TRPM8-channel-expressing HEK293 cells

HEK293 cells stably transfected with the human form of the TRPM8 channel show a concentration-dependent increase in intracellular calcium concentration, apparent as an increased calcium fluorescence over baseline values, following administration of the TRMP8 channel-selective agonist icilin to cells loaded with the Ca^{2+}fluorophore Calcium 4 (Figure 4.1). This effect of icilin is highly likely to be TRPM8-mediated as it was reversed in a concentration-dependent manner (Figure 4.2) by the TRPM8 channel-selective blocker AMTB (Lashinger et al., 2008) and was absent in untransfected HEK293 cells.

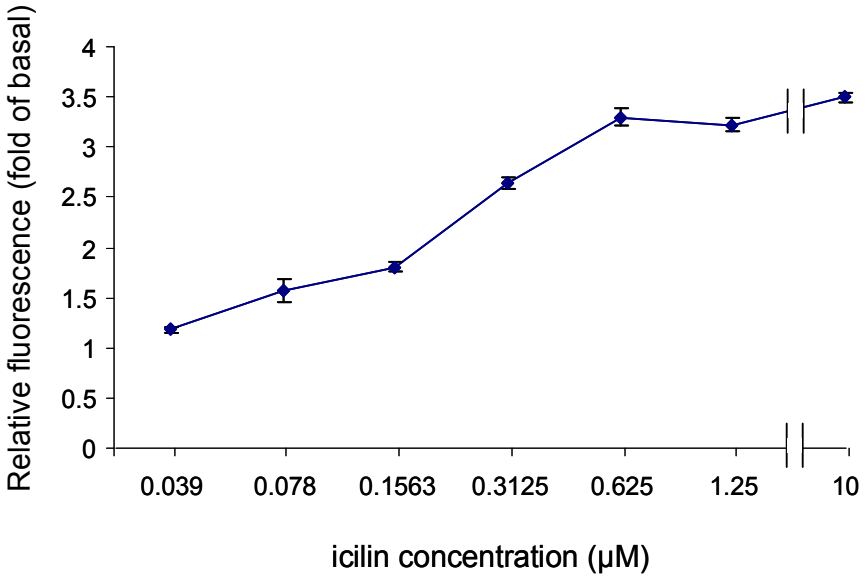

Figure 4.1: *Concentration-response curve for icilin showing intracellular Ca^{2+} concentration (expressed in relative fluorescence units as fold of basal) in stable TRPM8 channel-expressing HEK293 cells.*

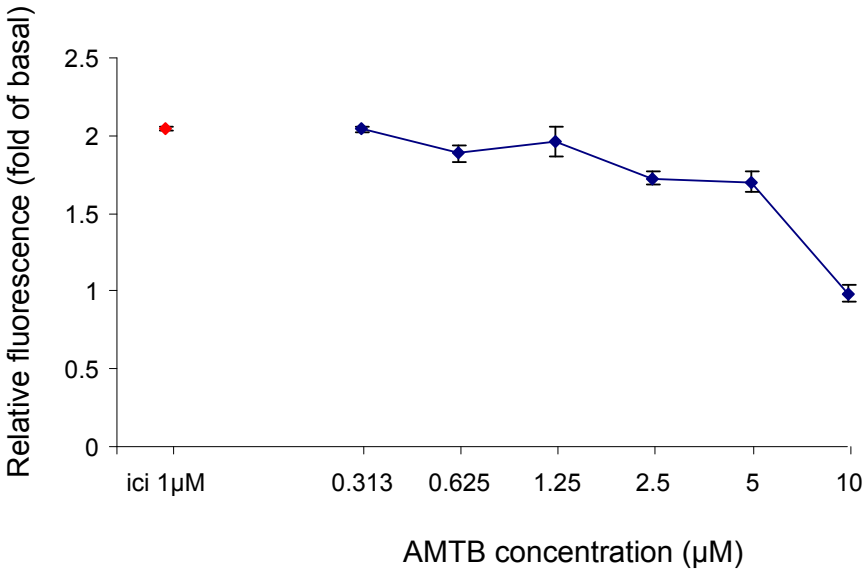

Figure 4.2: *Concentration-response curve showing a decrease in intracellular Ca^{2+} concentration (expressed in relative fluorescence units) in stable TRPM8 channel-expressing HEK293 cells, following administration of increasing concentrations of the TRPM8 channel blocker AMTB (dark blue) co-administered with icilin (1μM), compared to icilin (1μM) alone (red).*

4.2.2 In vitro cell model studies showing that activation of the 5-HT_{1B} receptor results in enhancement of icilin-induced TRPM8 channel responses

In order to ascertain whether 5-HT_{1B} receptor activation modulates TRPM8 channel function, calcium fluorometry studies were carried out on HEK293 cells stably expressing the human TRPM8 channel and transiently transfected with the 5-HT_{1B} receptor. Application of the TRPM8 channel agonist icilin resulted in concentration-dependent increased levels of intracellular Ca^{2+} as assessed by calcium fluorometry (Figure 4.3). When the selective 5-HT_{1B} receptor agonist CP 94253 (5-propoxy-3-(1,2,3,6-tetrahydro-4-pyridinyl)-1H-pyrrolo[3,2-b]pyridine; 3μM) was co-administered with icilin, intracellular Ca^{2+} levels were significantly higher than with icilin alone at 1.25μM, 2.5μM and 5μM ($p<0.05$; Student's paired t-test) (Figure 4.3.). CP 94253 on its own (at both 1.5μM and 3μM) did not result in a significant increase of intracellular Ca^{2+} over baseline levels (Figure 4.3). It has been shown that CP 94253 is a potent and selective 5-HT_{1B} receptor agonist and its functional activity has been demonstrated in both in vivo and in vitro experiments (Fish et al., 1999; Koe et al., 1992; Lee et al., 2002).

Significantly increased intracellular Ca^{2+} levels were also observed within seconds following co-administration of icilin plus CP 94253 using a real time fluorometry protocol (Figure 4.4). Importantly, this increment in intracellular Ca^{2+} was abolished in the presence of the selective 5-HT_{1B} receptor antagonist SB 224289 in addition to icilin and CP 94253 (Figure 4.4). SB 224289 has been shown to be a high affinity, selective antagonist/inverse agonist at 5-HT_{1B} receptors (Gaster et al., 1998; Roberts et al., 2001).

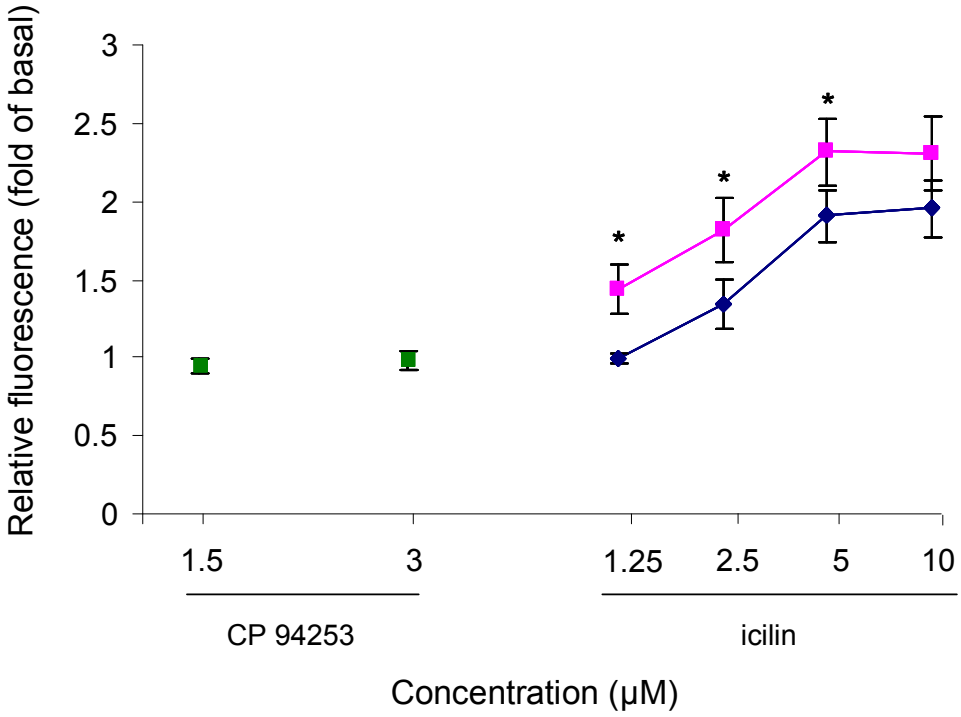

Figure 4.3: *Concentration-response curve showing intracellular Ca^{2+} concentration (expressed in relative fluorescence units) in stable TRPM8 channel-expressing HEK293 cells transfected with the 5-HT_{1B} receptor, following icilin application alone (1.25μM, 2.5μM, 5μM and 10 μM; dark blue) or co-administration of icilin (1.25μM, 2.5μM, 5μM and 10 μM) and the 5-HT_{1B} receptor agonist CP 94253 (3μM; pink). CP 94253 alone (1.5μM or 3μM; green) did not result in an increase of fluorescence over baseline values. These experiments were carried out in late passage TRPM8-HEK293 cells, where the absolute potency of TRPM8 agonists was reduced compared to that in early passage cells, but the relative facilitation by 5-HT_{1B} receptor activation was maintained (n=3, $p<0.05$; Student's paired t-test) .*

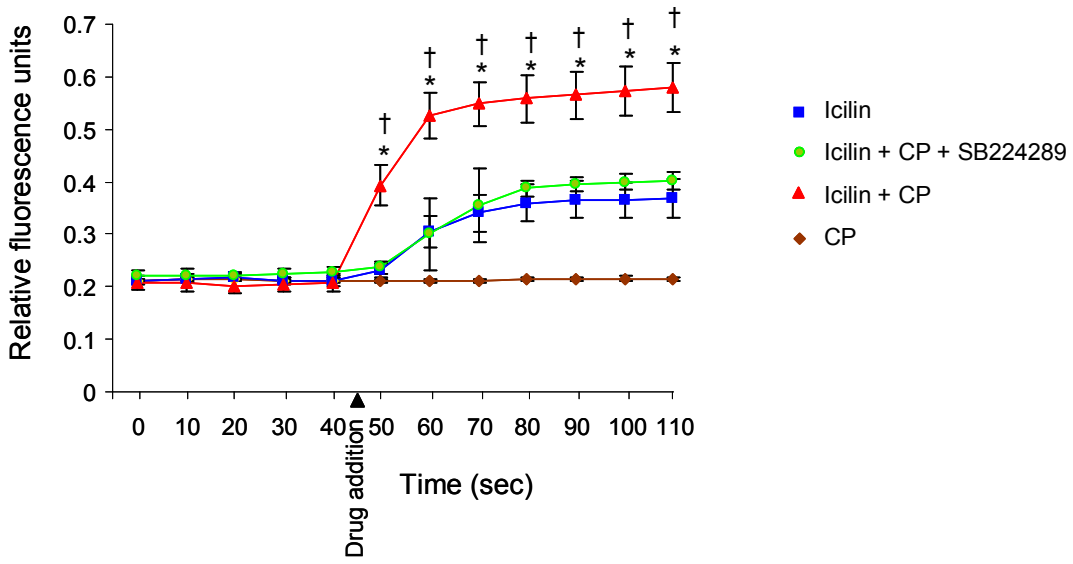

Figure 4.4: *Real-time calcium fluorometry (expressed in relative fluorescence units) indicating intracellular Ca^{2+} concentration in stable TRPM8-channel-expressing HEK293 cells, transfected with the 5-HT_{1B} receptor, following application of icilin (0.7μM; blue), the 5-HT_{1B} receptor agonist CP 94253 (2.5μM; brown), simultaneous application of icilin plus CP 94253 (red) and simultaneous application of icilin, CP 94253 and the selective 5-HT_{1B} receptor antagonist SB224289 (5μM; green). Co-administration of icilin and CP 94253 resulted in a significant increase in fluorescence compared to icilin alone ($p<0.05$; Student's paired t-test) and this effect was abolished when icilin and CP 94253 were administered in conjuction with SB224289 ($p<0.05$; Student's paired t-test). Administration of CP 94253 alone did not result in increased fluorescence over baseline values. Values are means ± SEM from 5 separate determinations.*

4.2.3 TRPM8 channels and 5-HT_{1B} receptors physically interact in an in vitro cell model

In order to investigate whether the functional interaction between the 5-HT_{1B} receptor and the TRPM8 channel might be mediated by way of a physical association between the channel and the receptor, co-immunoprecipitation studies were carried out using TRPM8 channel-expressing HEK293 cells transfected with FLAG-tagged 5-HT_{1B} receptor. Figure 4.5 shows representative immunoblots from TRPM8 channel immunoprecipitation on the left and anti-FLAG immunoprecipitation (the reciprocal strategy, used to isolate FLAG-tagged 5-HT_{1B} receptor) on the right. Top panels confirm specific isolation of TRPM8 channel (left) and 5-HT_{1B} receptor (identified as protein bands of

appropriate molecular weight) captured by immunoprecipitation antibodies, compared to same-species non-immune IgG controls. The bottom-left panel represents FLAG-5-HT_{1B} receptor co-immunoprecipitated by specific antibody against the TRPM8 channel, with no FLAG-5-HT_{1B} receptor co-immunoprecipition evident in control non-immune IgG pull-downs, indicating that the 5-HT_{1B} receptor specifically interacts with the TRPM8 channel. This was further confirmed by using monoclonal anti-FLAG antibody to pull down the 5-HT_{1B} receptor and probing of immunoprecipitates with anti-TRPM8 channel antibody. The bottom-right panel represents TRPM8 channel co-immunoprecipitating with anti-FLAG (5-HT_{1B} receptor) antibody. The greater density of the band isolated with anti-FLAG antibody, compared to non-immune IgG, further supports the idea that TRPM8 channel specifically interacts with FLAG-5-HT_{1B} receptor.

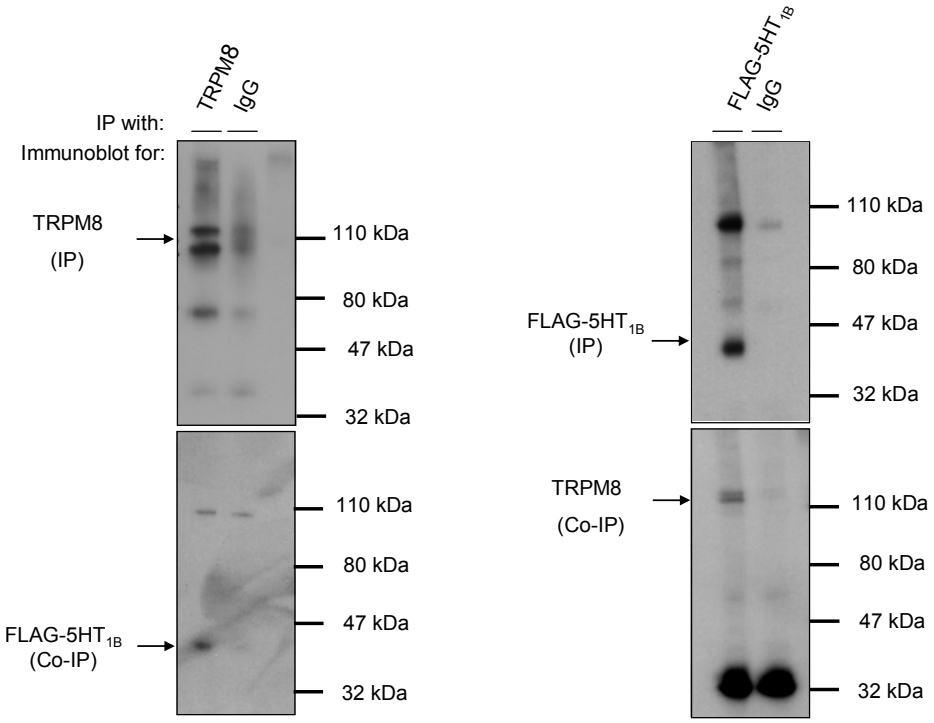

Figure 4.5: *Investigation of co-immunoprecipitation of the TRPM8 channel and the 5-HT_{1B} receptor. Stable TRPM8 channel-expressing HEK293cells were transfected with FLAG-tagged 5-HT_{1B} receptors. Immunoprecipitation with a TRPM8 antibody (left) or with anti-FLAG antibody (right) pulled down levels of 5HT_{1B} receptor and TRPM8 channel (Co-IP), respectively, which were not identified in non-immune IgG controls.*

As the 5-HT_{1B} receptor appears to form a complex with the TRPM8 channel, we investigated whether 5-HT_{1B} receptor-mediated signalling might influence the channel. The TRPM8 channel is negatively modulated by PKC and PKA phosphorylation (Abe et al., 2006; De Petrocellis et al., 2007; Premkumar et al., 2005) but positively modulated by phosphatidylinositol 4,5-bisphosphate; PIP_2 (Liu and Qin, 2005; Rohacs et al., 2005). The 5-HT_{1B} receptor signals predominantly through pathways that do not cause cleavage of PIP_2. Indeed, one pathway from this receptor is activation of PLD (unpublished work from the lab) and the PLD product, phosphatidic acid, can activate phosphatidyl inositol 5-kinase to increase PIP_2 levels (Cockcroft, 2009). Thus one possibility is that PLD and increased PIP_2 levels may be involved in the 5-HT_{1B} receptor facilitation of TRPM8 channel function.

4.2.4 Evidence suggesting that 5-HT_{1B} receptor-mediated enhancement of TRPM8 channel activation may be due to enhancement of PIP_2 levels by a PLD1-dependent mechanism

Calcium fluorometry studies were carried out to investigate the signalling involved in the 5-HT_{1B} receptor-mediated enhancement of TRPM8 activation by icilin, observed as increased intracellular Ca^{2+} levels. The possible role of PLD1 in mediating this effect was investigated using a range of inhibitors of PLD1 and modulators of other relevant signalling events in stable TRPM8 channel-expressing HEK293 cells transiently transfected with the 5-HT_{1B} receptor. Experiments were designed so that the effect of specific drugs when co-administered with icilin and the 5-HT_{1B} receptor agonist CP 94253 (2μM), was tested against three controls within each experiment: application of icilin (0.25μM), co-administration of icilin (0.25μM) plus the 5-HT_{1B} receptor agonist CP 94253 (2μM) and co-administration of icilin (0.25μM) and the specific drug tested. This experimental design allowed for the identification of any specific effects of the drugs tested on the 5-HT_{1B} receptor–mediated enhancement of intracellular Ca^{2+} levels through TRPM8 channel activation by icilin.

Figure 4.6 summarises the results from this set of experiments. Co-administration of the PLD1 inhibitors CAY10593 (VU0155069) 0.5 μM (Scott et

al., 2009) halopemide 4 μM (Scott et al., 2009) raloxifene 25 μM (Eisen and Brown, 2002) and calphostin C 2.5 μM (Sciorra et al., 2001) with icilin plus CP 94253 resulted in a statistically significant reduction of the enhancement in intracellular Ca^{2+} levels observed when co-administration of icilin plus CP 94253 was compared to application of icilin alone (Mann-Whitney U Test, $p<0.05$). Application of icilin plus PLD1 inhibitors did not result in significantly different intracellular Ca^{2+} levels compared to icilin alone.

Calphostin C acts as a potent PLD1 inhibitor (Sciorra et al., 2001), but following UV exposure this drug acts as a PKC inhibitor (Bruns et al., 1991). Although care was to taken to avoid UV exposure, a control using the PKC inhibitor, bisindolylmaleimide 1, BIM1 (Toullec et al., 1991) was carried out. Co-administration of BIM1 (2.5 μM) with icilin plus CP 94253 resulted in significantly increased intracellular Ca^{2+} levels compared to icilin alone (Mann-Whitney U Test, $p<0.05$) and these levels were not significantly different to those obtained with icilin plus CP 94253 (Figure 4.6). Application of icilin and BIM1 did not result in significantly different levels of intracellular Ca^{2+} than application of icilin alone (Figure 4.6). These results suggest that the enhancement in intracellular Ca^{2+} levels observed following the concurrent activation of the 5-HT_{1B} receptor and the TRPM8 channel is not mediated by PKC. This is consistent with evidence that PKC is a negative modulator of the channel (Abe et al., 2006; Premkumar et al., 2005).

Both phosphatidylinositol 4 and 5- kinases are required for PIP_2 synthesis, and the latter are activated by the PLD product, phosphatidic acid (Cockcroft, 2009; Liu and Qin, 2005; Rohacs et al., 2005). In order to assess the role of PI kinases in the downstream events following concurrent activation of the 5-HT_{1B} receptor and the TRPM8 channel, the effects of wortmannin were investigated. Wortmannin is a potent inhibitor of PI 3-kinases, but also with somewhat lower potency, of PI 4-kinases (Arcaro and Wymann, 1993). Co-administration of wortmannin with icilin plus CP 94253 to stable TRPM8-expressing HEK293 cells transfected with the 5-HT_{1B} receptor resulted in statistically significant lower levels of intracellular Ca^{2+} levels compared to those obtained when administering icilin and CP 94253 (Mann-Whitney U Test, $p<0.05$) (Figure 4.6).

Co-administration of wortmannin with icilin did not result in significantly different levels of intracellular calcium levels compared to administration of icilin alone (Figue 4.6). These results suggest that the increase in fluorescence observed when the 5-HT_{1B} receptor and the TRPM8 channel are simultaneously activated may be dependent on the activity of PI 4-kinase and which is consistent with a role for PIP_2 here.

In order to further assess the role of PIP_2 on this signalling pathway, we investigated the effects of drugs that modulate the activity of phospholipase C (PLC) which cleaves PIP_2 into diacylglycerol (DAG) and inositol 1, 4, 5 - trisphosphate (IP_3). Co-administration of the PLC activator, 2,4,6-trimethyl-N-[3-(trifluoromethyl)phenyl]benzenesulfonamide (m-3M3FBS) (Bae et al., 2003) (2.5 μM) with icilin plus CP 94253 to stable TRPM8 channel-expressing HEK293 cells transiently transfected with the 5-HT_{1B} receptor resulted in statistically significant lowered levels of intracellular Ca^{2+} , compared to administration of icilin plus CP 94253 (Mann-Whitney U Test, $p<0.05$) (Figure 4.6). These results suggest that the increase in fluorescence observed when the 5-HT_{1B} receptor and the TRPM8 channel are simultaneously chemically activated is abolished if PIP_2 is cleaved by PLC. Co-administration of m-3M3FBS (2.5 μM) with icilin also resulted in a significantly lower level of intracellular calcium following compared to administration of icilin alone (Mann-Whitney U Test, $p<0.05$) suggesting that PIP_2 is partially required ordinarily for the full extent of icilin-mediated activation of the TRPM8 channel (Figure 4.6).

Co-administration of the PLC inhibitor U-73122, ((1-[6-[[17-beta-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione) (Bleasdale et al., 1990) (10 μM) with icilin and CP resulted in a significant increase of intracellular Ca^{2+} levels compared to administration of icilin and CP (Mann-Whitney U Test, $p<0.05$) (Figure 4.6). Co-administration of the PLC inhibitor U-73122 (10 μM) with icilin did not result in significantly different levels of intracellular calcium compared to administration of icilin alone (Figure 4.6). This is consistent with the idea that inhibition of ongoing PIP_2 cleavage would increase PIP_2 levels and promote any role in modulating the TRPM8 channel.

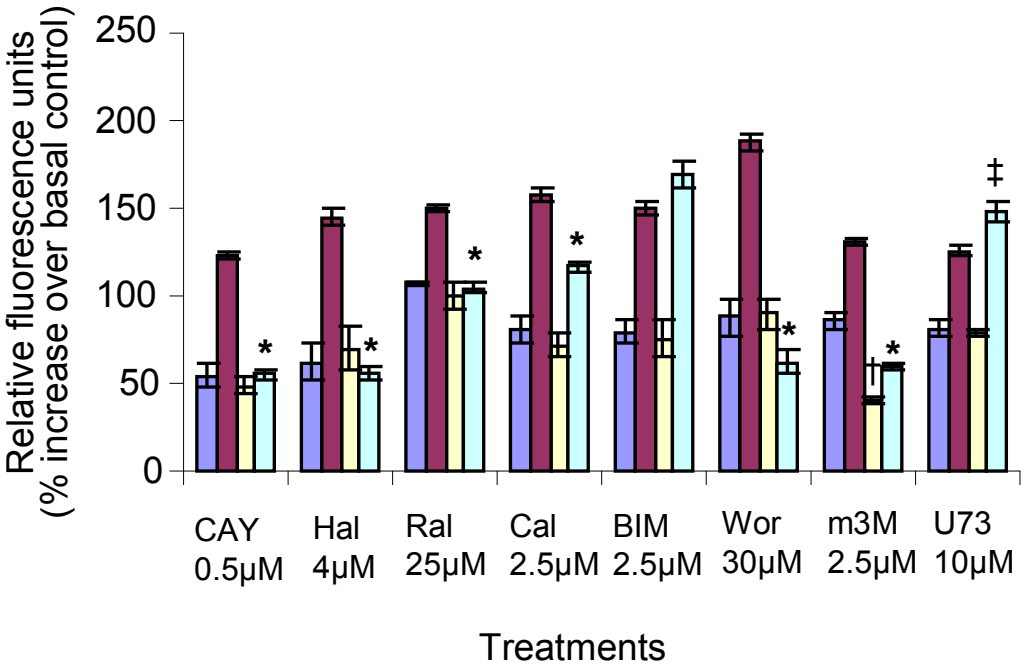

Figure 4.6: *Intracellular Ca^{2+} levels, as assessed by calcium fluorometry (expressed as percentage increase over basal control) following application of icilin 0.25μM (dark blue), icilin 0.25μM and CP 94253 2μM (dark red), drug treatment plus icilin (yellow) and drug treatment plus icilin and CP 94253 (light blue). The enhancement in Ca^{2+} levels over application of icilin alone observed when icilin is co-administered with CP 94253 was significantly reduced by concurrent application of PLD1 inhibitors CAY10593 (CAY), halopemide (Hal), raloxifene (Ral) and calphostin C (Cal) (but not the PKC inhibitor, bisindolymaleimide (BIM1). Similar significant reductions in icilin plus CP94253 responses (* $p<0.05$, Mann-Whitney U-Test) were seen with the PI kinase inhibitor, wortmannin (Wor) and the PLC activator, m-3M3FBS (m3M), which also attenuated responses to icilin alone († $p<0.05$). The PLC inhibitor U-73122 (U73) caused significant facilitation of responses to icilin plus CP94253 (‡ $p<0.05$, Mann-Whitney U-Test). Values are means ± SEM from 5 determinations.*

In order to obtain additional molecular evidence for a role of PLD and identify which isoform might be responsible, stable TRPM8 channel-expressing HEK293 cells were co-transfected with the 5-HT_{1B} receptor and either the empty vector pcDNA3.1, dominant negative (catalytically inactive) PLD1 or dominant negative (catalytically inactive) PLD2. The constructs [K898R] PLD1 and [K758R] PLD2 were kindly provided by Mike Frohman. Calcium fluorometry experiments were carried out in these cells to investigate levels of intracellular Ca^{2+} following administration of icilin (0.25 μM) and icilin (0.25 μM) plus CP 94253 (2 μM). Figure 4.7 illustrates the results from this set of experiments. Co-administration of icilin and CP94253 resulted in a highly statistically significant increase in intracellular calcium levels compared to icilin alone in cells transfected with pcDNA3.1 or with dominant negative-PLD2 ($p<0.01$, Mann-Whitney U-Test) (Figure 4.7). In cells transfected with dominant negative-PLD1, co-administration of icilin and CP94253 still resulted in a statistically significant increase in intracellular Ca^{2+} levels compared to icilin alone ($p<0.05$, Mann-Whitney U-Test) but this increase was significantly lower than the response observed in cells transfected with pcDNA3.1 ($p<0.05$, Mann-Whitney U-Test) (Figure 4.7). These observations specifically implicate PLD1 rather than PLD2 in the effect of the 5-HT_{1B} receptor on TRPM8 channel responses.

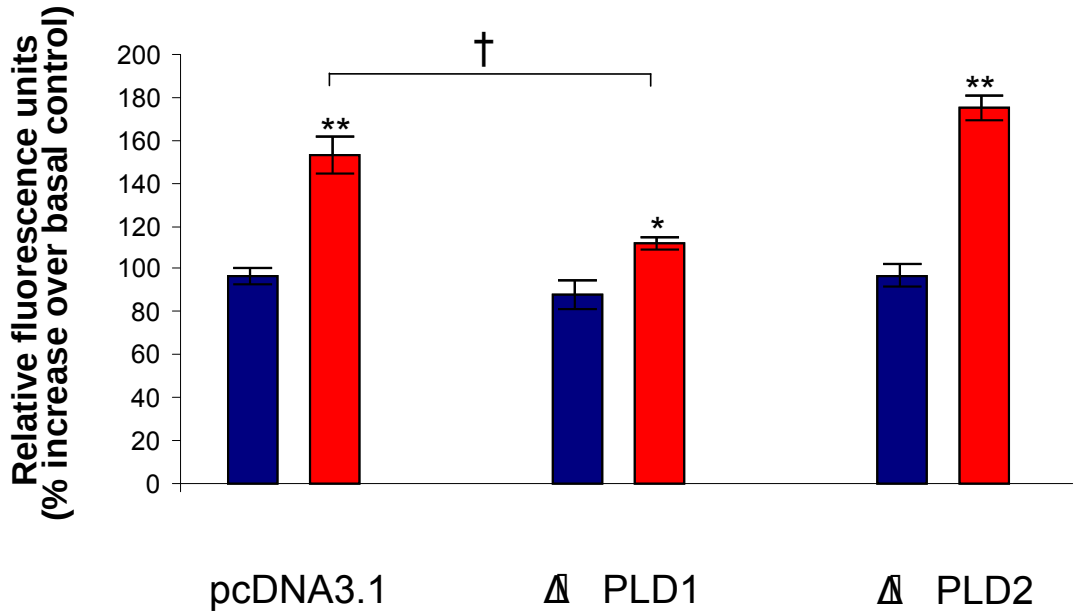

Figure 4.7: *Intracellular Ca^{2+} levels, as assessed by calcium fluorometry (expressed as percentage increase over basal control) following application of icilin 0.25μM (dark blue) or icilin 0.25μM plus CP 94253 2μM (red). Stable TRPM8 channel-expressing HEK293 cells were transiently transfected with the 5-HT_{1B} receptor and also transfected with either an empty vector (pcDNA3.1), dominant negative PLD1 or dominant negative PLD2. CP94253 caused significant facilitation of responses to icilin in cells transfected with pcDNA3.1 or dominant negative PLD2 (** $p<0.001$, Mann-Whitney U-Test and also to a lesser extent in cells with dominant negative PLD1 (* $p<0.05$). The enhancement of icilin-induced increases in intracellular Ca^{2+} levels due to CP94253 was significantly attenuated († $p<0.05$, Mann-Whitney U-Test) in cells transfected with the dominant negative PLD1 compared to pcDNA3.1 controls. Values are means ± SEM from 10 separate determinations.*

4.2.5 TRPM8 channels and the 5-HT_{1B} receptor physically interact with PLD1 in an in vitro cell model

Further co-immunoprecipitation studies were then carried out to investigate whether PLD1 physically interacts with the TRPM8 channel and/or the 5-HT_{1B} receptor in vitro. Stable TRPM8 channel-expressing HEK293 cells were transiently transfected with HA-tagged PLD1 and FLAG-tagged 5-HT_{1B} receptor.

Figure 4.8 shows representative blots from this set of co-immunoprecipitation studies. Immunoblots from anti-FLAG immunoprecipitation (used to isolate FLAG-tagged 5-HT_{1B} receptor) are shown in the left panels, immunoblots from TRPM8 channel immunoprecipitation are shown in the third from left panels and immunoblots from anti-HA immunoprecipitation (used to isolate HA-tagged PLD1) are shown in the second and fourth from left panels. Top panels confirm specific isolation of the primary pull down targets FLAG 5-HT_{1B} receptor, TRPM8 channel and HA-PLD1 in each case compared to same-species non-immune IgG controls.

The lower panels display blots for co-immunoprecipitated proteins. The high density band in the bottom-left panel represents HA-PLD1 co-immunoprecipitate pulled down with anti-FLAG, with only a faint corresponding band pulled down with control non-immune IgG, indicating that PLD1 specifically interacts with the 5-HT_{1B} receptor. In order to confirm this result, HA-PLD1 was isolated by using anti-HA antibody. Although immunoblotting of anti-HA immunoprecipitates with anti-FLAG identified a band of appropriate molecular weight suggesting 5-HT_{1B} receptor binding to HA-PLD1 pulldowns, IgG controls probed with anti-FLAG also produced a faint band.

The high density band in third-from-left bottom panel represents HA-PLD1 co-immunoprecipitate pulled down with the TRPM8 channel, with only a much lower density corresponding band pulled down with control non-immune IgG, indicating that PLD1 specifically interacts with the TRPM8 channel. In order to confirm this result, HA-PLD1 was isolated by using anti-HA antibody. Immunoblotting of anti-HA co-immunoprecipitates with anti-TRPM8 channel antibody identified a band of appropriate molecular weight suggesting TRPM8

binding to HA-PLD1 pulldowns, IgG control pulldowns probed with anti-TRPM8 channel antibody produced only a faint band of roughly the same molecular weight.

Taken together these co-immunoprecipitation studies suggest that both the TRPM8 channel and the 5-HT_{1B} receptor physically interact with PLD1 in an in vitro cell model and may together form some kind of regulatory complex.

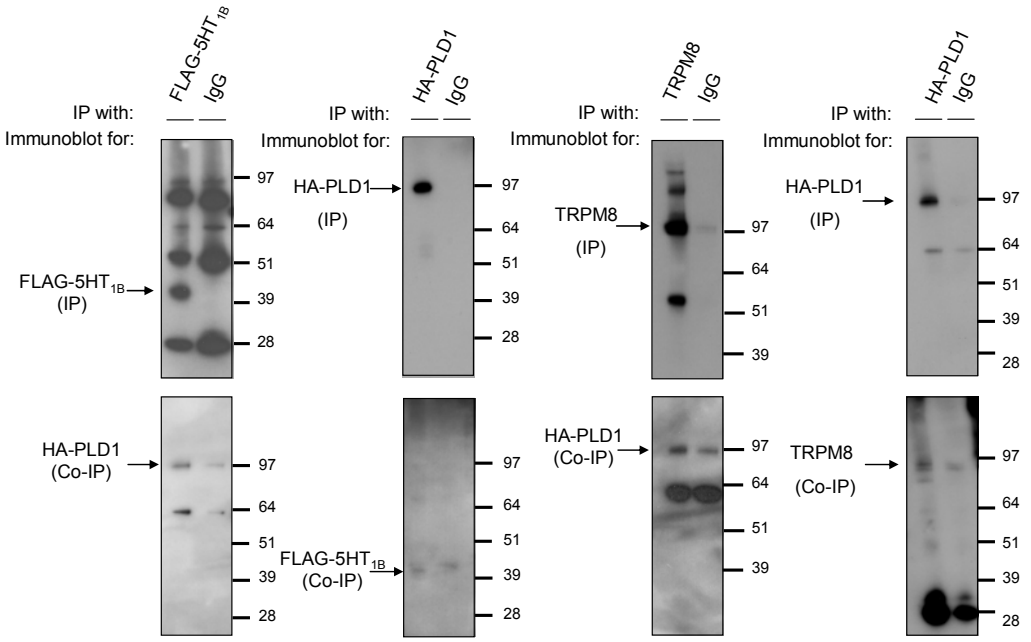

Figure 4.8: *Investigation of co-immunoprecipitation of PLD1 with FLAG-tagged 5-HT_{1B} receptor and TRPM8 channel. Stable TRPM8 channel-expressing HEK293cells were transfected with FLAG-tagged 5-HT_{1B} receptor and HA-tagged PLD1. Immunoprecipitation was carried out with anti-FLAG antibody, anti-TRPM8 channel antibody or anti-HA antibody: (labels above each column of panels). The upper panels in each case show blots carried out for the primary pulldown target to show the success of the specific pulldown procedures. The lower panels in each case show probing of these pulldowns for associated (co-immunoprecipitated) proteins, HA-PLD1, FLAG-5-HT_{1B} receptor and TRPM8 channel. Taken together these results suggest that both the 5-HT_{1B} receptor and the TRPM8 channel interact with PLD1, presumably in some form of regulatory signalling complex.*

4.2.6 Expression of 5-HT_{1B} receptor protein in rat (naïve and CCI) DRG and spinal cord

In order to investigate the patterns of expression of the 5-HT_{1B} receptor in chronic neuropathic pain states, Western immunoblotting was carried out in whole lysates obtained from DRGs and spinal cord ipsilateral to injury in CCI rats at peak sensitivity compared to contralateral and naïve samples (n=3 per group). A 5-HT_{1B} receptor-immunoreactive band was found at an appropriate molecular weight but no statistically significant difference was observed between treatments (expressed in relative intensity expressed as a proportion of GAPDH expression) (ANOVA, $p>0.05$) (Figure 4.9).

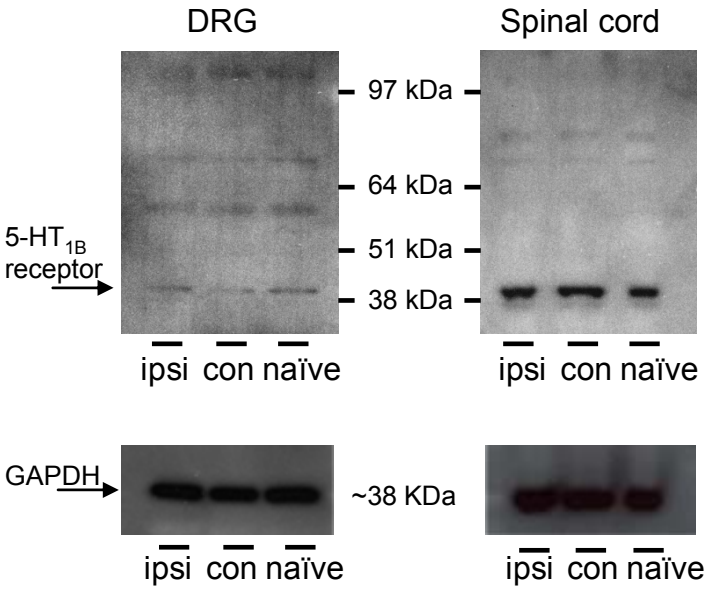

Figure 4.9: *Western immunoblots of dorsal root ganglia (DRG) and spinal cord whole lysates from CCI neuropathic pain model rats (ipsilateral and contralateral to injury) and naïve rats (n=3 per group) incubated with a 5-HT_{1B} receptor antibody (upper panels). Lower panels represent incubation with antibody for the housekeeping enzyme GAPDH. No statistically significant differences between treatments (calculated from data expressed as relative grey scale intensity relative to GAPDH levels) were identified.*

4.2.7 Calcium imaging of 5-HT_{1B} receptor modulation of TRPM8 channel responses in cultured DRG cells

Calcium imaging studies prepared with the assistance of Andrew Allchorne (in collaboration with Dr John Curtis) were carried out on DRG neuron cultures in order to investigate 5-HT_{1B}-receptor mediated modulation of TRPM8 channel activation by icilin in native systems. Experiments were designed to compare a drug treatment with an initial application of icilin alone. The first icilin application was used both to identify icilin-responsive cells (only a small fraction, 5-10% of the cultured DRG cells) and also to provide a response against which the effects of the treatment were compared. Figure 4.10 provides an illustration of a typical icilin response. The two treatments within each experiment were separated by a wash in order to return fluorescence levels to baseline. Areas under the curve were calculated for each of the two treatments and compared using a Wilcoxon Signed Rank Test (GraphPad Prism 4).

Simultaneous application of icilin and the 5-HT_{1B} receptor agonist CP 94253 resulted in significantly higher intracellular Ca^{2+} levels compared to application of icilin alone ($p<0.05$, $n = 19$) (Figures 4.10, 4.11 and 4.13). In order to elucidate whether a second icilin application resulted in increased fluorescence, areas under the curve produced from two consecutive icilin applications separated by a wash were compared. No significant difference was observed between two successive icilin applications (Figure 4.12 and 4.13, $n = 12$).

In order to investigate if the CP 94253-mediated enhancement of TRPM8 channel activation by icilin was mediated by PLD1, the PLD1 inhibitor calphostin C was co-administered with icilin and CP 94253. No significant difference was observed between areas under the curve obtained following co-administration of icilin, CP 94253 and calphostin C ($n = 14$) compared to those obtained for icilin alone, i.e. no enhancement of the TRPM8 channel activation caused by icilin was observed (Figure 4.13). Co-administration of BIM1 (in order to control for PKC inhibition, as described above) with icilin and CP 94253 resulted in a significantly increased response compared to that of icilin alone ($p<0.05$, $n = 17$), although the absolute magnitude of the responses did appear less than those due to icilin plus CP 94253 alone (Figure 4.13).

Single administration of CP 94253, calphostin C or BIM1 to icilin-responsive cells did not result in any discernible effects, defined as a 10% variation over the baseline.

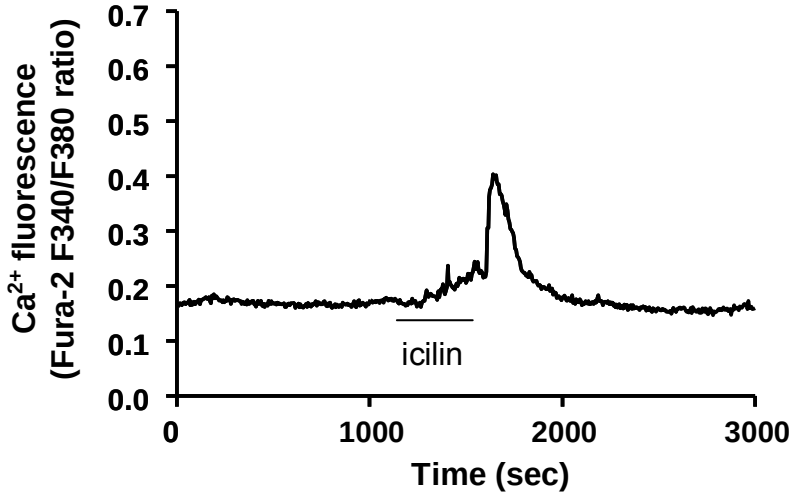

Figure 4.10: *Trace from a calcium imaging recording from a single DRG cell showing a typical response to application of icilin (2.5 μM). Each recording period represented 5 sec. Similar responses to the selective TRPM8 channel activator icilin were seen in approximately 5-10% of DRG cells.*

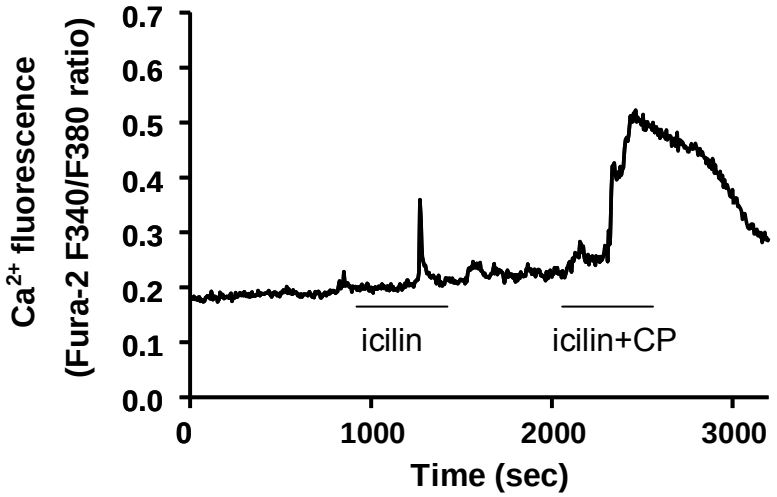

Figure 4.11: *Trace from a calcium imaging recording from a single DRG cell showing a typical response to application of icilin (2.5 μM) followed by a response to icilin (2.5 μM) plus CP 94253 (3 μM).*

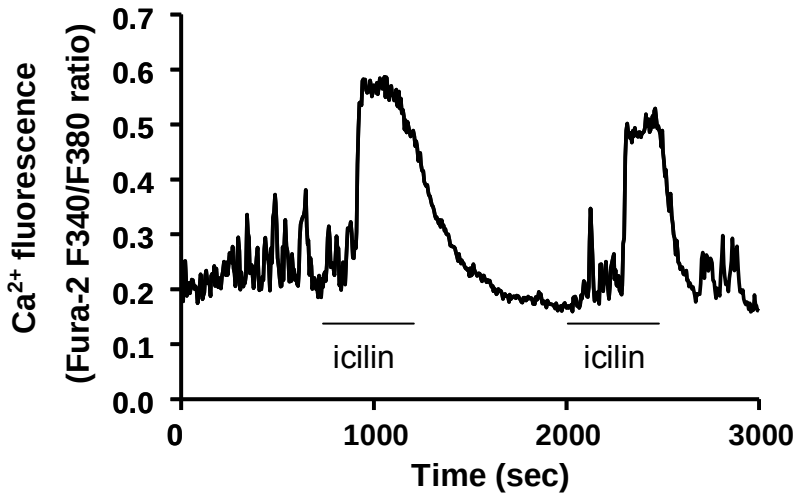

Figure 4.12: *Trace from a calcium imaging recording from a single DRG cell showing two typical consecutive responses to icilin (2.5 µM).*

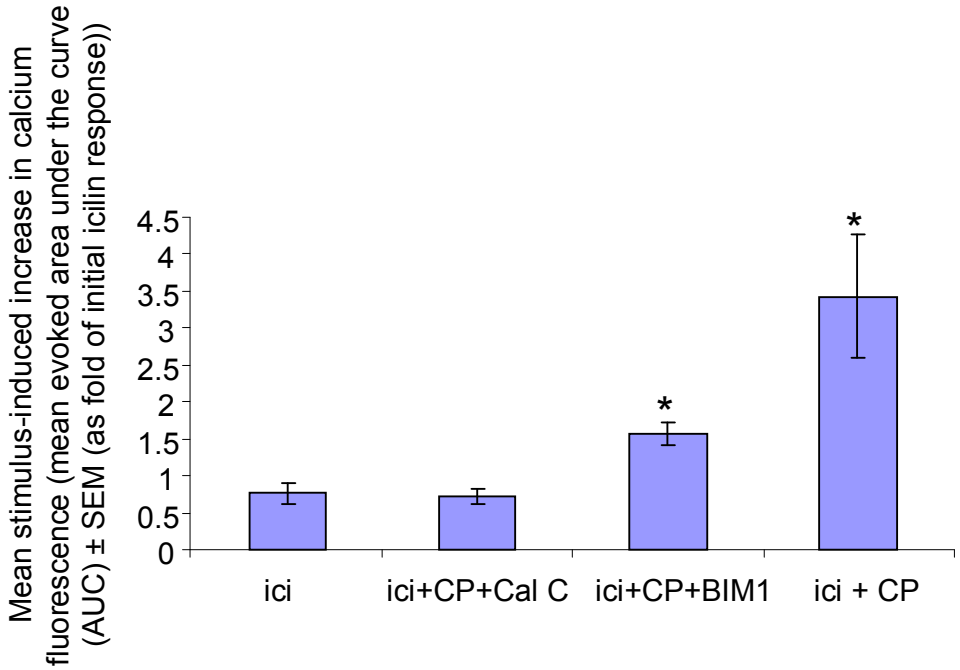

Figure 4.13: *Mean stimulus-induced increase in intracellular Ca^{2+} fluorescence resulting from various treatments expressed as fold increase in area under the curve (AUC) of excess response expressed as mean ratio ± SEM compared to initial icilin response. Experiments were carried out in 3 coverslips/treatment, with an average number of icilin responsive DRG cells of 15.5 (±1.6) per treatment. Treatments were: icilin (2.5 µM) plus CP 94523 (3 µM), icilin (2.5 µM) plus CP 94253 (3 µM) and calphostin C (3 µM), icilin (2.5 µM) plus CP 94253 (3 µM) and BIM1 (2.5 µM) or straightforward repeat of icilin (2.5 µM).*

4.2.8 TRPM8 channels and 5-HT1B receptors physically interact in vivo

In order to investigate whether the functional interaction between the 5-HT_{1B} receptor and the TRPM8 channel observed in calcium imaging experiments carried out in DRG neuron cultures is correlated with a direct physiological association between the channel and the receptor, co-immunoprecipation studies were carried out on spinal cord lysates from naive rats. This was carried out in spinal cord rather than DRG tissue because inordinately high numbers of animals would be required for immunoprecipitation experiments on DRG.

Figure 4.14 illustrates the results from this study. Immunoprecipitation with a TRPM8 channel antibody pulled down 5-HT_{1B} receptor immunoreactivity (co-IP), identified as a dense band around 50kDa, which was not present in non-immune IgG controls. Following incubation of the 5-HT_{1B} receptor primary antibody with a specific blocking peptide (the antigen to which it was raised; right panels), Western blotting immunodetection of 5-HT_{1B} receptor was abolished, demonstrating specificity of the antibody for the target protein.

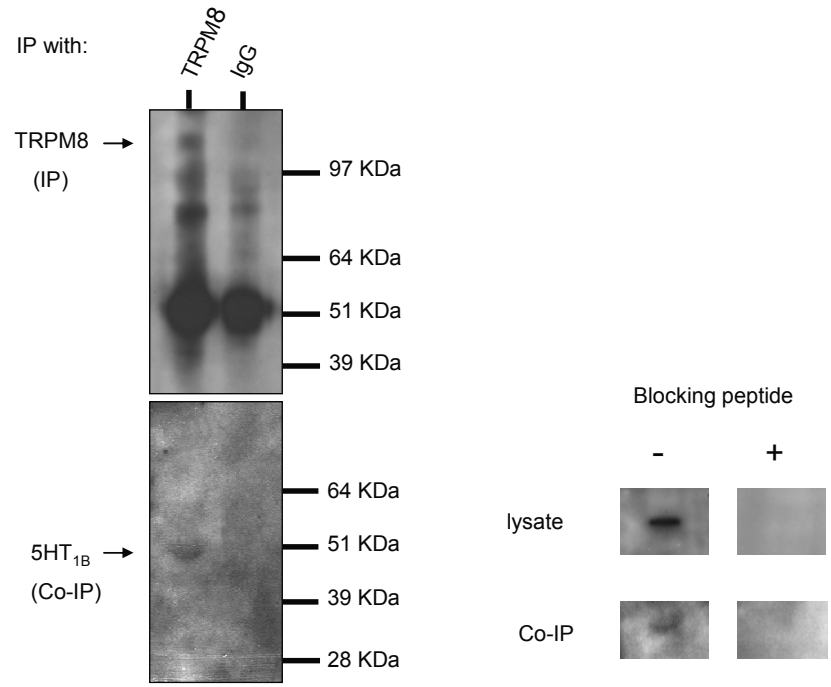

Figure 4.14: *Investigation of co-immunoprecipitation of the TRPM8 channel and the 5-HT$_{1B}$ receptor in spinal cord homogenates obtained from naïve rats. Results are typical of three separate experiments. Immunoprecipitation with an anti-TRPM8 channel antibody pulled down levels of 5HT$_{1B}$ receptor (co-IP), identified as a dense band between the 51kDa and 39 kDa markers, which was not observed in non-immune IgG controls. When a blocking peptide against the epitope recognised by the 5-HT$_{1B}$ receptor primary antibody (right panels) was preincubated with the antibody (pre-absorbtion control), Western blotting immunodetection of the 5-HT$_{1B}$ receptor was abolished, showing specificity of the antibody.*

4.2.9 Behavioural reflex investigation of 5-HT$_{1B}$ receptor-mediated modulation of TRPM8 channel mediated analgesia in the CCI model of neuropathic pain

The 5-HT$_{1B}$ receptor agonist, CP 94253 (2.5 nmol) was injected intrathecally together with the TRPM8 channel agonist icilin (2.5 nmol) in CCI rats at peak behavioural sensitivity in order to investigate whether the previously reported analgesic effect of intrathecally administered icilin was modulated by 5-HT$_{1B}$ receptor activation. The effect of A) icilin (2.5 nmol) alone, B) icilin (2.5 nmol) plus CP 94253 (2.5 nmol) or C) CP 94253 (2.5 nmol) alone on the mechanical and thermal hyperalgesia exhibited by CCI rats at baseline is shown in Figures

4.15 and 4.16 respectively and expressed as mean paw withdrawal thresholds (in mN/mm^2, assessed by von Frey filaments) and mean paw withdrawal latency (in seconds, assessed by Hargreaves' thermal test).

Intrathecal administration of icilin (at a submaximal dose) resulted in a statistically significant difference between ipsilateral baseline mechanical withdrawal threshold values and ipsilateral post-injection values (Friedman test, $p<0.05$; followed by post-hoc Dunnett's test which indicated a significant difference at 15 mins post-injection). Additionally ipsilateral and contralateral values were not significantly different at 25 mins post-injection (Wilcoxon test, $p>0.05$) (Figure 4.15 A). However, thermal withdrawal latency did not reach statistically significant difference between ipsilateral baseline and post-injection values (One-way repeated measures ANOVA, $p>0.05$) and ipsilateral and contralateral values remained significantly different at all time points (Wilcoxon test, $p<0.05$) (Figure 4. 16 A).

Intrathecal administration of icilin and CP 94253 together did not result in a statistically significant difference between ipsilateral baseline and post-injection mechanical withdrawal thresholds (Figure 4.15 B). However, there was a statistically significant difference between baseline and post-injection thermal withdrawal latency (One-way repeated measures ANOVA, $p<0.05$, with post-hoc Dunnett's test showing a significant difference at 15 mins post, injection). Additionally, ipsilateral and contralateral values were not significantly different at 15 and 25 mins post-injection (Figure 4.16 B).

Intrathecal administration of CP 94253 alone did not show statistically significant differences between baseline and post-injection values either on mechanical withdrawal thresholds or on thermal withdrawal latency (Figure 4.15 C and 4.16 C).

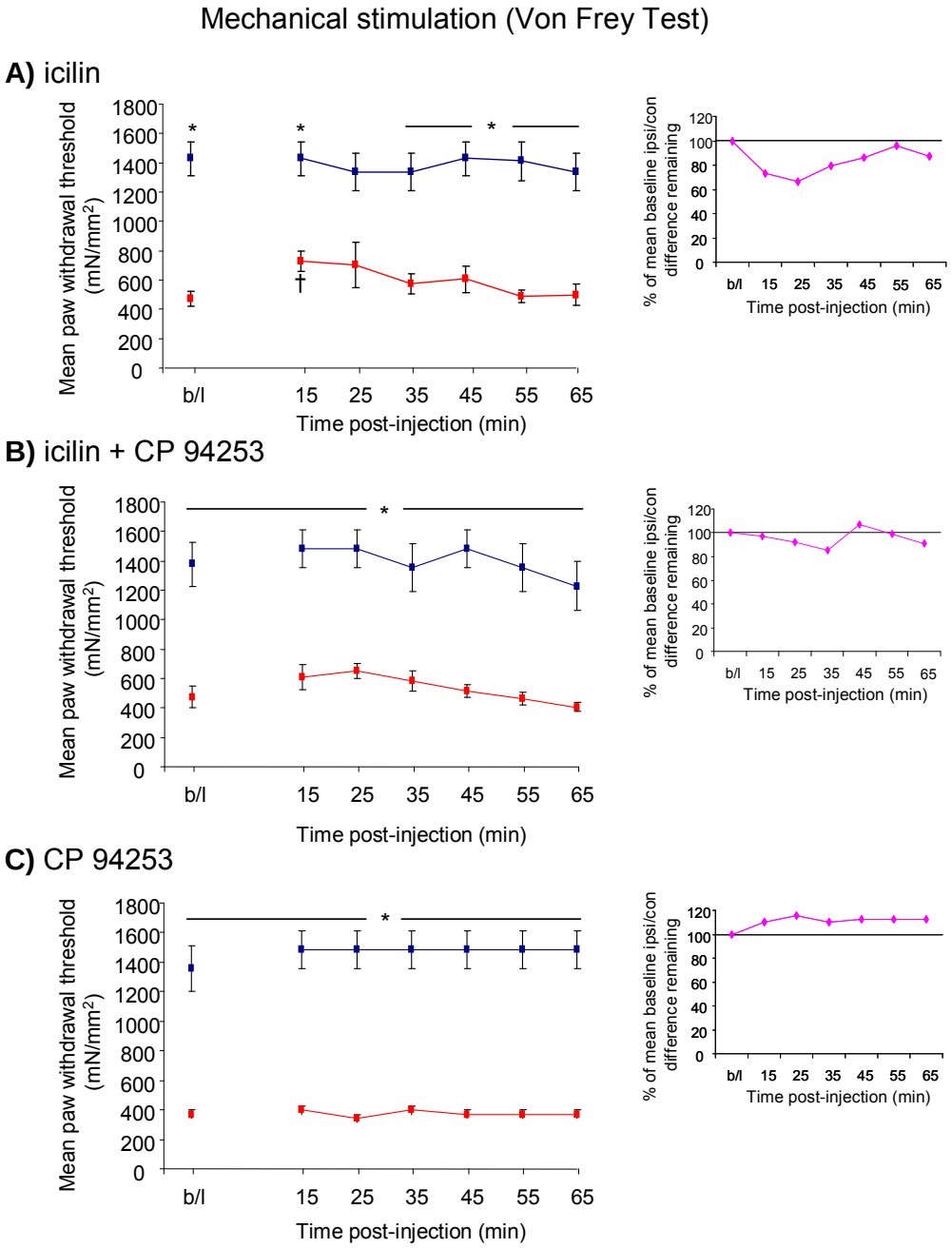

Figure 4.15: *Effects of intrathecal administration of A) icilin (2.5 nmol); B) the selective 5-HT$_{1B}$ receptor agonist CP 94253 (2.5 nmol) co-administered with icilin (2.5 nmol) and C) CP 94253 (2.5 nmol) alone on the mechanical hypersensitivity developed in the ipsilateral paw (red) compared to the contralateral paw (blue) in rats at peak behavioural sensitisation following CCI surgery (n=6). Data show mean paw withdrawal thresholds (in mN/mm2) ± SEM. (*) denotes statistically significant difference between ipsilateral and contralateral values at individual time points (Wilcoxon signed rank test). (†) denotes statistically significant difference between ipsilateral baseline values and post-injection values (Friedman test). The inset graphs show the percentage of mean baseline difference between ipsilateral and contralateral values remaining over time.*

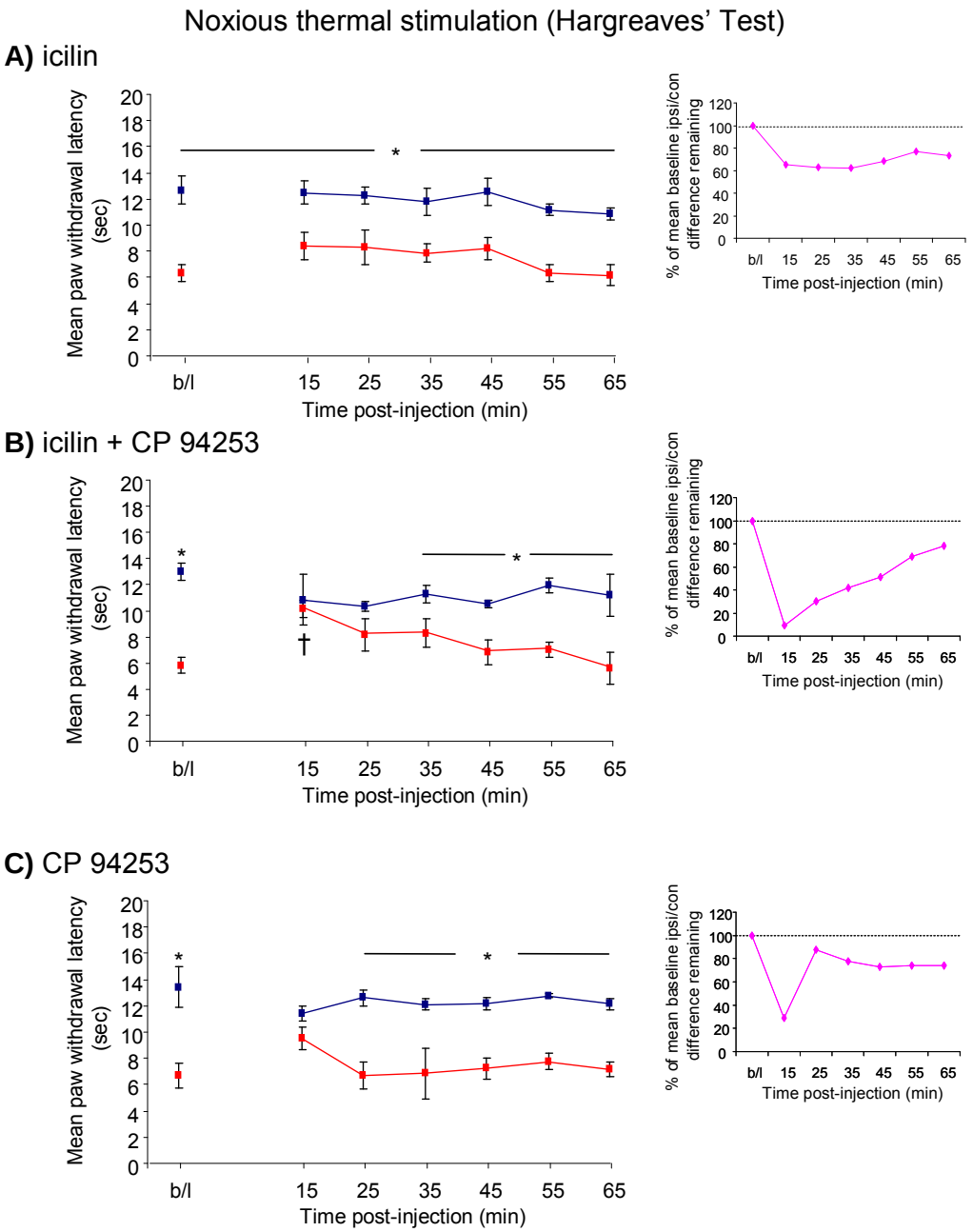

Figure 4.16: *Effects of intrathecal administration of A) icilin (2.5 nmol); B) the selective 5-HT$_{1B}$ receptor agonist CP 94253 (2.5 nmol) co-administered with icilin (2.5 nmol) and C) CP 94253 (2.5 nmol) alone on thermal hyperalgesia developed in the ipsilateral paw (red) compared to the contralateral paw (blue) in rats at peak behavioural sensitisation following CCI surgery (n=6). Data show mean paw withdrawal latency (in seconds) ± SEM. (*) denotes statistically significant difference between ipsilateral and contralateral values at individual time points (Paired t-test). (†) denotes statistical significant difference between ipsilateral baseline values and post-injection values (One-way repeated measures ANOVA test). The inset graphs show the percentage of mean baseline difference between ipsilateral and contralateral values remaining over time.*

Figures 4.17 and 4.18 show mechanical withdrawal thresholds and thermal withdrawal latencies in CCI rats at peak behavioural sensitivity following administration of A) icilin alone (2.5 nmol) B) icilin (2.5 nmol), plus CP 94253 (2.5 nmol) and the PLD1 inhibitor, calphostin C (0.65 nmol) and C) calphostin C (0.65 nmol) alone.

Intrathecal administration of icilin and CP 94253 in the presence of calphostin C did not result in a statistically significant difference between ipsilateral baseline and post-injection mechanical withdrawal thresholds (Friedman test, $p>0.05$) (Figure 4.17 B). Additionally, there was no statistically significant difference between baseline and post-injection thermal withdrawal latency (One-way repeated measures ANOVA, $p>0.05$) (Figure 4.18 B, contrasting with the significant differences previously observed in the case of icilin plus CP 94253 (Figure 4.16B)).

Intrathecal administration of calphostin C alone did not show statistically significant differences between baseline and post-injection values either on mechanical withdrawal thresholds or on thermal withdrawal latency (Figure 4.17 C and 4.18 C).

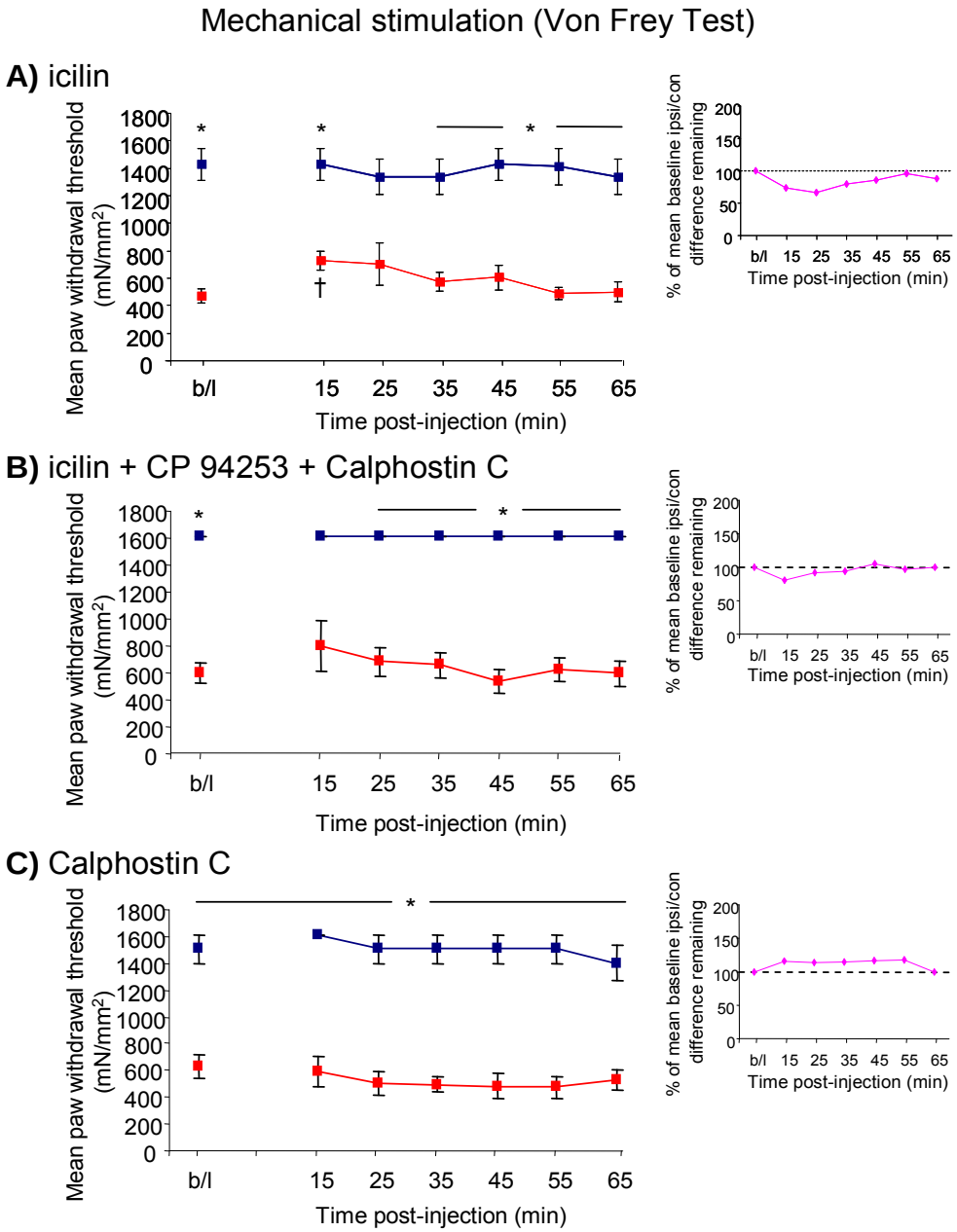

Figure 4.17: *Effects of intrathecal administration of A) icilin (2.5 nmol); B) the selective PLD1 inhibitor Calphostin C (0.65 nmol) co-administered with icilin (2.5 nmol) and CP 94253 (2.5 nmol) and C) Calphostin C (0.65 nmol) alone on the mechanical hypersensitivity developed in the ipsilateral paw (red) compared to the contralateral paw (blue) in rats at peak behavioural sensitisation following CCI surgery (n=6). Data shows mean paw withdrawal threshold (in mN/mm^2) ± SEM. (*) denotes statistically significant difference between ipsilateral and contralateral values at individual time points (Wilcoxon signed rank test). (†) denotes statistically significant difference between ipsilateral baseline values and post-injection values (Friedman test). The inset graphs show the percentage of mean baseline difference between ipsilateral and contralateral values remaining over time.*

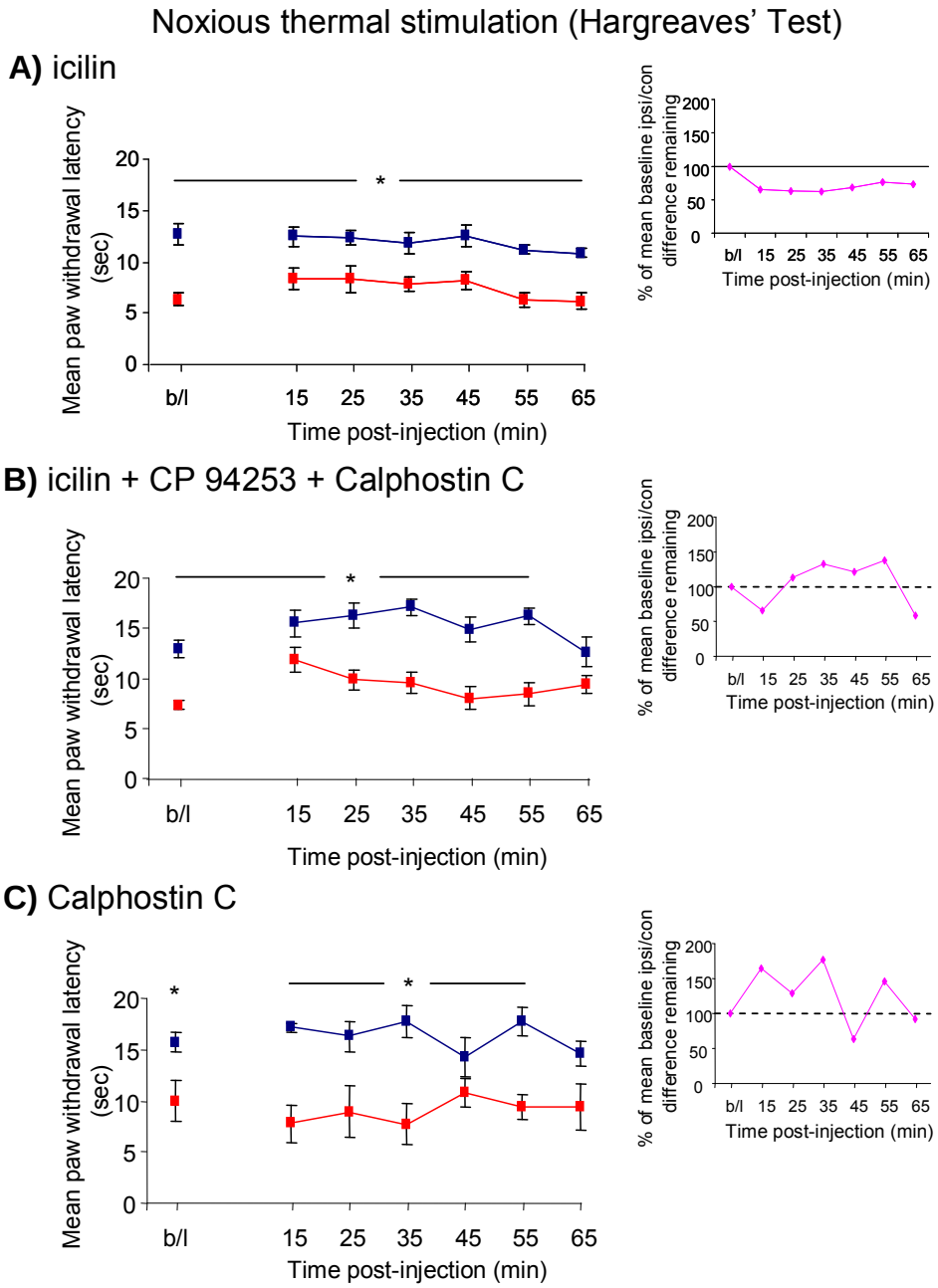

Figure 4.18: *Effects of intrathecal administration of A) icilin (2.5 nmol); B) the selective PLD1 inhibitor Calphostin C (0.65 nmol) co-administered with icilin (2.5 nmol) and CP 94253 (2.5 nmol) and C) Calphostin C (0.65 nmol) alone on thermal hyperalgesia developed in the ipsilateral paw (red) compared to the contralateral paw (blue) in rats at peak behavioural sensitisation following CCI surgery (n=6). (*) denotes statistically significant difference between ipsilateral and contralateral values at individual time points (Paired t-test). (†) denotes statistical significant difference between ipsilateral baseline values and post-injection values (One-way repeated measures ANOVA test). Data shows mean paw withdrawal latency (in seconds) ± SEM. The inset graphs show the percentage of mean baseline difference between ipsilateral and contralateral values remaining over time.*

4.3 Discussion

Cooling and topical menthol have been historically used as analgesics. Recent advances in molecular cloning have made possible the identification of a member of the transient receptor potential family of channels, TRPM8, as a transducer in cool temperature detection and also as the mediator involved in cooling analgesia in pathophysiological (sensitised) pain states (Proudfoot et al., 2006). However, activation of the TRPM8 channel under normal, unchallenged physiological conditions does not result in alteration of nociceptive thresholds. This suggests that the TRPM8 channel must be subject to a mechanism of facilitatory modulation in chronic pain states that enables its analgesic effect upon activation. TRPM8 channel expression has been noted to increase in DRG neurons ipsilateral to injury in the CCI model of neuropathic pain (Frederick et al., 2007; Proudfoot et al., 2006). However, no alterations in TRPM8 channel expression were reported in the SNL model of nerve injury or in a model of inflammation (Katsura et al., 2006; Obata et al., 2005). Thus, increased expression of the TRPM8 channel in sensory neurons does not appear a sufficient mechanism to explain the reversal of nociceptive sensitisation observed following TRPM8 channel activation in chronic pain states.

Serotonin (5-HT) has been suggested to play an important role in pain processing following injury. 5-HT is released by platelets and mast cells following tissue injury, inflammation and also after nerve injury (Anden and Olsson, 1967; Dray, 1995; Vogel et al., 2003). Several 5-HT receptors have been identified in sensory neurons, with different, even antagonistic effects in pain processing (see section 4.1.4.). 5-HT_{1B} receptors appear to mediate antinociceptive effects and their pattern of expression in peripheral afferents is very similar to that of TRPM8 channels. Thus, 5-HT_{1B} appeared like a good candidate to investigate as a potential mediator in the facilitation of TRPM8 channel activation in sensitised pain states.

4.3.1 Chemically mediated TRPM8 channel opening is facilitated by activation of the 5-HT1B receptor

In order to investigate our hypothesis that TRPM8 channel activity is modulated by 5-HT_{1B} receptor activation a number of *in vitro* studies were carried out. Administration of the TRPM8 channel selective agonist icilin to HEK293 cells stably transfected with the human form of the TRPM8 channel showed a concentration-dependent increase in intracellular calcium concentration and this effect was reversed by the selective TRPM8 channel-selective blocker AMTB and was absent in untransfected HEK293 cells, confirming specificity of this action through the TRPM8 channel. Co-administration of icilin and the 5-HT_{1B} receptor-selective agonist CP 94253 to HEK293 cells stably transfected with TRPM8 and transiently transfected with the 5-HT_{1B} receptor, resulted in intracellular Ca^{2+} levels that were significantly higher than with icilin alone. Administration of CP 94253 without icilin did not result in an increase in intracellular Ca^{2+}. Moreover, the increment in intracellular Ca^{2+} associated with co-administration of icilin and CP 94253 was abolished in the presence of the selective 5-HT_{1B} receptor antagonist SB 224289, confirming the specificity of this action through the 5-HT_{1B} receptor.

Our results from the calcium imaging experiments carried out in dissociated adult rat DRG neurons also suggested a facilitatory action of the 5-HT_{1B} receptor on TRPM8 function in native systems, as the increase in calcium fluorescence associated with icilin was potentiated when icilin was co-administered with CP 94253. Further calcium imaging studies should aim to confirm the specificity of this action using 5-HT_{1B} antagonists. Additionally, since we propose that this mechanism might operate in chronic pain states through the mediation of serotonin, calcium imaging studies in DRG cultured neurons investigating the role of serotonin on TRPM8-mediated calcium entry, using 5-HT_{1B} receptor agonists and antagonists, would also be valuable.

Recent studies have suggested a modulatory role of serotonin receptors on TRPV1 channel activity. Activation of spinal 5-$HT_{2A/2C}$ receptors with the selective agonist DOI ((6)-2,5-dimethoxy-4-iodoamphetamine) resulted in an increase in capsaicin-evoked release of SP-like immunoreactivity in the rat

dorsal horn, which was partially reversed by the 5-HT_{2A} receptor antagonist ketanserin (Kjorsvik et al., 2003). The 5-HT_{2A} receptor has been recently shown to be expressed in TRPV1-positive primary sensory neurons (Van et al., 2009). Additionally, calcium imaging and whole-cell patch-clamp studies on cultured DRG neurons from newborn rats showed that 5-HT potentiated the increases of $[Ca^{2+}]$i, current and depolarizing responses induced by capsaicin, protons and noxious heat (Ohta et al., 2006). TRPV1 function is also potentiated by 5-HT in colon sensory neurons from rats and mice and this has been suggested as a mechanism mediating visceral hypersensitivity (Qin et al., 2010; Sugiuar et al., 2004). The 5-HT_{2A} receptor has been shown to be involved in pro-nociceptive actions and to be implicated in inflammatory pain (its expression being upregulated in inflammation), whereas 5-HT_{1B} has been associated with anti-nociception (Abbott et al., 1997; el-Yassir et al., 1988; Gjerstad et al., 1997; Kayser et al., 2007; Obata et al., 2000; Okamoto et al., 2002). These divergent actions appear to be furthered through the functional association of 5-HT_{2A} and 5-HT_{1B} with a typically nociceptive (TRPV1) and analgesic (TRPM8) system, respectively.

Western immunoblotting was carried out in whole lysates obtained from DRGs and spinal cord ipsilateral to injury in CCI rats at peak sensitivity compared to contralateral and naïve samples, but no statistically significant difference in the expression of the 5-HT_{1B} receptor was observed between treatments. Upregulation of the 5-HT_{1B} receptor has been described in inflammatory pain models (Liu et al., 2005; Wu et al., 2001) but to our knowledge no changes in the expression patterns of this receptor have been reported in models of neuropathic pain.

Extensive trials were carried out with a variety of commercially available 5-HT_{1B} receptor antibodies in order to try and identify the subpopulation of DRG cells in which the receptor is expressed (and to investigate possible co-localisation with the TRPM8 channel) using double-label immunofluorescence histochemistry. Despite lengthy evaluation of different fixation, signal

amplification and incubation techniques it was not possible to identify an adequate reagent and protocol.

The functional interaction between the 5-HT_{1B} receptor and the TRPM8 channel described above might be mediated by a physical association between the channel and the receptor, as suggested by the results from co-immunoprecipitation studies on HEK293 cells transfected with the TRPM8 channel and the 5-HT_{1B} receptor. Physical interaction of the TRPM8 channel and the 5-HT_{1B} receptor was also observed in spinal cord homogenates from naïve rats, suggesting that this might be relevant *in vivo*. No previous reports have identified a physical interaction between a GPCR and a TRP channel.

4.3.2 5-HT_{1B} receptor-mediated facilitation of TRPM8 channel activation may be due to a PLD1-dependent mechanism

Recent studies have unravelled mechanisms of TRPM8 modulation, both inhibitory and facilitatory. The TRPM8 channel appears to be negatively modulated by PKC and PKA phosphorylation (Abe et al., 2006; Bavencoffe et al., 2010; De Petrocellis et al., 2007; Premkumar et al., 2005). On the other hand, TRPM8 activity is positively modulated by phosphatidylinositol 4, 5-bisphosphate, PIP_2 (Liu and Qin, 2005; Rohacs et al., 2005).

As our study suggested that the 5-HT_{1B} receptor appears to form a complex with the TRPM8 channel, we investigated whether 5-HT_{1B} receptor-mediated signalling might influence the channel. The 5-HT_{1B} receptor signals predominantly through pathways that do not cause cleavage of PIP_2. Indeed, one pathway from this receptor is activation of PLD (unpublished work from the lab) and PLD can activate phosphatidylinositol 5-kinase to increase PIP_2 levels (Cockcroft, 2009).

Co-administration of the PLD1 inhibitors CAY10593 (VU0155069), halopemide, raloxifene or calphostin C with icilin and the 5-HT_{1B} receptor agonist, CP 94253 resulted in the abolition of the enhancement in intracellular Ca^{2+} levels observed when co-administration of icilin plus CP 94253 was compared to application of icilin alone. These results were further confirmed in calcium imaging studies carried out in DRG cultured neurons where calphostin C, but not BIM-1 resulted in intracellular calcium responses to icilin plus CP

95243 that were not significantly different from those obtained by application of icilin alone.

Recent studies have identified a regulatory role of histamine receptors and metabotropic glutamate receptors on TRPC3 channel activity that is mediated by PLD (Glitsch, 2010; Kwan et al., 2009).

There are two mammalian specific PLD isoforms, PLD1 and PLD2, encoded by two PLD genes, each one with two splice-variants and each isoform appears to be involved in specific cellular processes (Hammond et al., 1997; Liscovitch et al., 2000). In order to identify which PLD isoform might be responsible for the observed 5-HT_{1B} receptor-mediated enhancement of TRPM8 channel activity, fluorometry studies were carried out in stable TRPM8 channel-expressing HEK293 cells co-transfected with the 5-HT_{1B} receptor and either the empty vector pcDNA3.1, dominant negative (catalytically inactive) PLD1 or dominant negative (catalytically inactive) PLD2. In cells transfected with the dominant negative PLD1 (but not the dominant negative construct), the icilin-induced increase in calcium fluorescence was significantly lower than the response observed in cells transfected with pcDNA3.1, suggesting that PLD1 rather than PLD2 is involved in the effect of the 5-HT_{1B} receptor on TRPM8 channel responses.

Further co-immunoprecipitation studies were carried out to investigate whether PLD1 physically interacts with the TRPM8 channel and/or the 5-HT_{1B} receptor in vitro. Our results suggest that PLD1 might bind to both the channel and the receptor. Previous studies have provided evidence for physical interactions of PLD with several GPCRs (Barclay, 2010; Bhattacharya et al., 2004; Koch et al., 2003). However, to our knowledge this is the first time that a regulatory signalling complex between a GPCR, a TRP channel and PLD has been described. PLD hydrolyses phosphatidylcholine to produce phosphatidic acid and choline (Liscovitch et al., 2000). PLD can result in activation of PIP 5-kinase which, in turn, is involved in PIP_2 synthesis (Cockcroft, 2009). Since PIP_2

is known to have a positive modulatory action in TRPM8 activity we carried out a number of in vitro experiments to investigate this pathway (Figure 4.19).

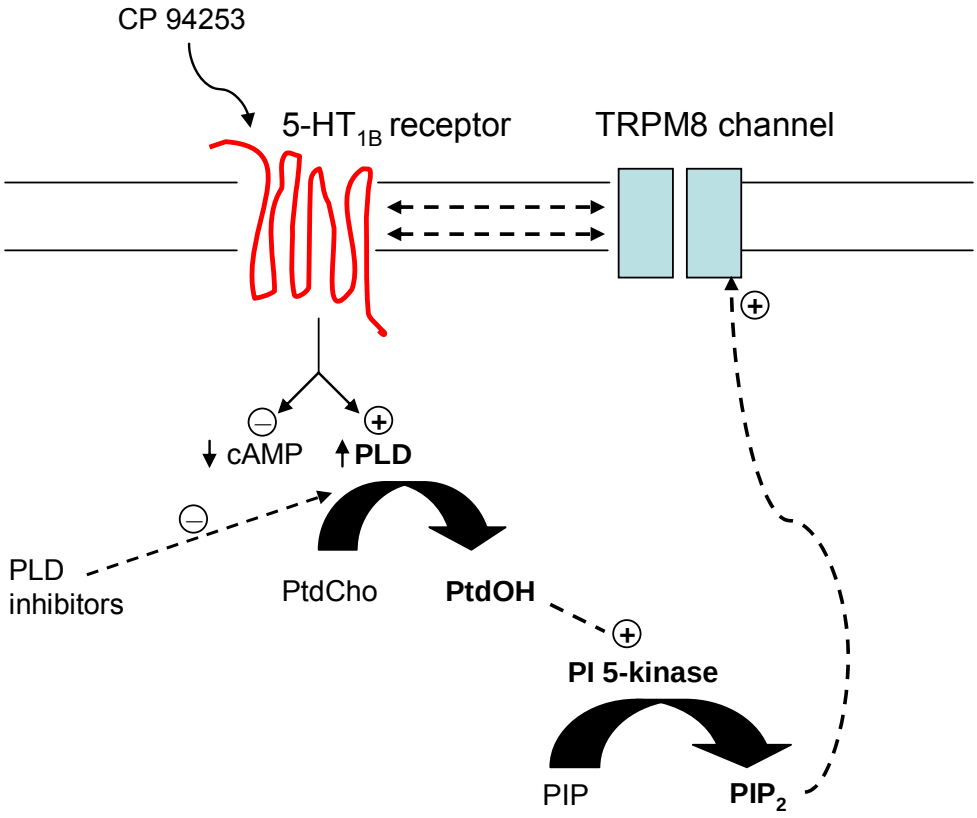

Figure 4.19: *Diagram illustrating the mechanism of interaction between the 5-HT_{1B} receptor and the TRPM8 channel, through PLD activation and increased PIP_2 levels. All processes relating to lipids will occur within the plasma membrane but the diagram has been expanded to aid clarity.*

In order to assess the role of PI kinases in the downstream events following concurrent activation of the 5-HT_{1B} receptor and the TRPM8 channel, the effects of wortmannin, an inhibitor of PI 3-kinases and PI 4-kinases were investigated using calcium fluorometry on stable TRPM8 channel-expressing HEK293 cells transiently transfected with the 5-HT_{1B} receptor. Wortmannin reversed the enhancement of intracellular Ca^{2+} levels obtained when administering icilin and CP 94253. Although wortmannin inhibits both PI 3-kinases and PI 4-kinases, studies using a low dose of wortmannin, which inhibits PI 3-kinases but not PI 4-

kinases, suggested that the inhibition of PIP_2 synthesis is mainly dependent on the activity of PI 4-kinase (Liu and Qin, 2005).

The role of PIP_2 in this signalling pathway was investigated using drugs that modulate the activity of phospholipase C (PLC) which cleaves PIP_2 into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). Co-administration of the PLC activator, m-3M3FBS with icilin plus CP 94253 to stable TRPM8 channel-expressing HEK293 cells transiently transfected with the 5-HT_{1B} receptor resulted in abolition of the increase in levels of intracellular calcium levels observed with administration of icilin plus CP 94253 compared to icilin alone. These results suggest that the increase in fluorescence observed when the 5-HT_{1B} receptor and the TRPM8 channel are simultaneously chemically activated is abolished if PIP_2 is cleaved by PLC. Interestingly, co-administration of m-3M3FBS with icilin also resulted in a significantly lower level of intracellular calcium compared to administration of icilin alone, suggesting that PIP_2 is partially required ordinarily for the full extent of icilin-mediated activation of the TRPM8 channel. Indeed, previous reports have suggested that PIP_2 acts constitutively as a positive regulator of TRPM8 channel activity (Liu and Qin, 2005; Rohacs et al., 2005).

Co-administration of the PLC inhibitor U-73122 with icilin and CP resulted in a significant increase of intracellular Ca^{2+} levels compared to administration of icilin and CP. This is consistent with the idea that inhibition of ongoing PIP_2 cleavage would increase PIP_2 levels and promote any role of PIP_2 in modulating the TRPM8 channel.

4.3.3 The role of 5-HT_{1B} receptor activation on TRPM8 channel-mediated analgesia in the CCI model of neuropathic pain

TRPM8 channel activation, using peripherally (topically) or centrally (intrathecally) applied TRPM8 channel agonists menthol and icilin in the CCI model of chronic neuropathic pain, results in a reduction of sensitised reflex pain behaviours (Proudfoot et al., 2006). In order to assess any modulatory influences of concurrent 5-HT_{1B} receptor activation on TRPM8 channel-mediated analgesia, we assessed effect of intrathecal co-administration of the 5-HT_{1B} receptor agonist, CP 94253 and the TRPM8 channel agonist icilin (compared to

administration of either icilin or CP 94253 alone) in CCI rats at peak behavioural sensitivity.

Although intrathecal administration of icilin (at a submaximal dose) resulted in a statistically significant difference between ipsilateral baseline mechanical withdrawal threshold values and ipsilateral post-injection values, thermal withdrawal latency did not reach statistically significant difference between ipsilateral baseline and post-injection values. This is probably a reflection of a sub-optimal dose of icilin being deliberately used in these experiments (Proudfoot et al., 2006).

Intrathecal administration of icilin and CP 94253 resulted in no significant difference between baseline and post-injection thermal withdrawal latency and between ipsilateral and contralateral values at particular time points post-injection. Intrathecal administration of CP 94253 alone did not show statistically significant differences between baseline and post-injection values either on mechanical withdrawal thresholds or on thermal withdrawal latency. This suggests that the reversal of sensory sensitisation observed with co-administration of icilin and CP 94253 is not solely the result of 5-HT_{1B} activation. Although previous studies have suggested an antinociceptive role for spinal 5-HT_{1B} receptors (el-Yassir et al., 1988; Gjerstad et al., 1997; Liu et al., 2007) this effect was not obvious at the dose required here to apparently facilitate TRPM8 channel activation.

In order to assess the role of PLD1 in the possible modulation of 5-HT_{1B} receptor activation on TRPM8 channel-mediated analgesia we assessed mechanical withdrawal thresholds and thermal withdrawal latencies in CCI rats at peak behavioural sensitivity following intrathecal co-administration of icilin, CP 94253 and the PLD1 inhibitor, calphostin C, compared to administration of icilin or calphostin C alone. Intrathecal administration of icilin and CP 94253 in the presence of calphostin C did not result in a statistically significant difference between ipsilateral baseline and post-injection mechanical withdrawal thresholds and there was no statistically significant difference between baseline and post-injection thermal withdrawal latency. This suggests the involvement of PLD1 in

the reversal of thermal behavioural sensitisation in CCI rats observed when there is concurrent activation of the 5-HT_{1B} receptor and the TRPM8 channel.

Taken together, these observations on nociceptive behavioural reflex assessments following intrathecal drug administration are consistent with the idea that activation of 5-HT_{1B} receptors can facilitate TRPM8 channel responses in vivo through a route involving PLD. However, further studies using a range of icilin doses and the administration of 5-HT_{1B} antagonist drugs would be required to confirm and fully develop these findings.

4.3.4 Conclusion

The results arising from this chapter provide evidence for a positive modulatory role of the 5-HT_{1B} receptor on TRPM8 channel activity, involving the actions of PLD1 and PIP_2 together with demonstration of a physical complex in both in vitro systems that brings the TRPM8 channel, the 5-HT_{1B} receptor (and PLD1) into direct contact. This could provide a therapeutic target for the development of novel analgesic protocols in the treatment of chronic pain states.

Chapter 5: SUMMARY AND CONCLUSION

Chronic neuropathic pain represents a major clinical concern for both human and animal patients. The mechanisms underlying chronic neuropathic pain states have not been fully elucidated and treatment remains problematic, reflecting a need for novel therapeutic targets to enable development of new improved analgesics. In animals, these issues are further complicated by the lack of objective pain assessment tools for both the rigorous characterisation of pain states and evaluation of the efficacy of current and novel therapeutic strategies.

Quantitative Sensory Testing (QST) has recently emerged as a preferred tool for monitoring pain in a clinical setting, as it provides a standardised, objective methodology, which does not rely on the clinician's experience. Quantification of nociceptive thresholds provides a measure of pain hypersensitivity (hyperalgesia and allodynia), which develops after surgery or is associated with chronic pain states (e.g. chronic lameness) (references reviewed by Vinuela-Fernandez et al., 2007). Moreover, QST has been shown to be reliable in measuring analgesic efficacy in a variety of pain models, validating the sensitivity of the method sensitivity and thus paving the way or its use in assessing the efficacy of novel analgesic drugs (e.g. KuKanich et al., 2005; Slingsby et al., 2001). One of the aims of this study was to develop a novel method for the assessment of hoof compression thresholds in horses suffering from equine laminitis, a disease associated with severe pain and debilitation. For that purpose, we used a novel hydraulically-powered feedback-controlled hoof tester. The results have shown that hoof compression thresholds (for limb withdrawal) of laminitic horses are consistently in the order of 50% lower than those of normal horses (a statistically significant alteration). This is consistent with previous studies reporting increased mechanical sensitivity on the hooves of laminitic horses. Moreover, the reliability of the method suggests that the new hoof tester could be used for the measurement of pain thresholds in both research and clinical contexts. Further studies should aim at establishing the method's sensitivity for the assessment of therapeutic efficacy of existing and novel treatments.

Laminitic horses often respond poorly to standard anti-inflammatory therapy. Although extensive research has been carried out to elucidate the pathogenesis of this disease, little is known about the neurobiology of the pain state associated with laminitis. Therefore, we aimed to characterise peripheral nerve damage that might have occurred as a consequence of the pathological changes associated with the disease. The results from this part of the study demonstrate for the first time that equine laminitis pain has a neuropathic component, evidenced by morphological changes in digital nerves innervating the hoof, including a significant reduction in the number of myelinated and unmyelinated fibres, and an increased expression of the nerve injury marker ATF3 and neuropeptide Y in DRG cells involved in hoof innervation. Our study was limited to the peripheral nervous system. Further studies should aim to uncover spinal cord mechanisms, for example investigating NMDA receptors and associated proteins, which might be involved in the maintenance of chronic laminitis pain.

Our findings are relevant to clinical strategies for the management of equine laminitis, as they suggest that the administration of anti-neuropathic analgesic agents may result in improved pain relief in horses suffering from refractory laminitis. Indeed, since the publication of our findings in the journal Pain (Jones et al., 2007), there have been reports in the veterinary literature advocating a modification in the approach to pain therapy in laminitic horses in order to address the neuropathic component of the disease (e.g. Driessen et al., 2010; Muir, 2010). Current pharmacological treatment for human patients suffering from neuropathic pain is still limited, either due to poor drug efficacy (with high numbers-needed-to-treat) or due to the associated adverse side effects (Collins et al., 2000). Although there is a lack of published clinical studies regarding the treatment of neuropathic pain in veterinary patients, the same limiting factors are likely to be relevant in this context (Grubb, 2010). Thus, there is a need for the identification of new targets and mechanisms that could lead the way to the development of more efficacious agents. The TRPM8 channel, which has been shown to exert an analgesic effect in laboratory models of chronic pain and human patients following its activation, appeared to be a prime candidate for further investigation.

Thus, the second component of the study reported the discovery and the investigation of a mechanism of TRPM8 channel modulation involving 5-HT_{1B} receptors. Using *in vitro* techniques we identified an increase in chemically-mediated TRPM8 channel activity when 5-HT_{1B} receptors were concurrently activated. Moreover, our results suggest that this facilitatory modulation might be mediated through a signalling complex formed between the TRPM8 channel, the 5-HT_{1B} receptor and PLD1, possibly involving downstream PIP_2 actions on TRPM8 channels. The uncovering of macromolecular signalling complexes formed between GPCRs, ion channels and enzymes generating second messengers has been described as a candidate mechanism for enhanced control achieved by spatial micro-organisation of key mediators (Laporte et al., 2001). An example of such a signalling complex involves the direct association between β_2 adrenergic receptors and the class C L-type calcium channel $Ca_v1.2$, in which, β_2 adrenergic receptor stimulation could increase neighbouring L-type channel activity (Davare et al., 2001). As the understanding of the regulatory role of these complexes expands, novel technologies to investigate protein:protein interactions have been and continue to be developed. Further studies should be carried out to validate the interaction between the TRPM8 channel and the 5-HT_{1B} receptor reported here. For example, the use of fluorescence resonance energy transfer (FRET) in cell lines (where the channel and the receptor carry distinct fluorescent-protein-tags), would allow an independent confirmation of the interaction.

In vivo results carried out in CCI rats at peak sensitivity suggest an enhancement of analgesic influence on thermal sensitivity associated with intrathecal co-administration of icilin and a 5-HT_{1B} receptor agonist, compared to administration of icilin alone. Further studies should aim to confirm these *in vivo* influences by investigating whether co-administration of a 5-HT_{1B} receptor antagonist with icilin and a 5-HT_{1B} receptor agonist reverses the enhancement in icilin-mediated analgesia. Since in our study, a suboptimal dose of icilin was used deliberately in order to maximise any observable effects of 5-HT_{1B} receptor activation on icilin-mediated analgesia, further dose finding studies should aim to

establish optimal doses of icilin and 5-HT_{1B} receptor agonist, in order to achieve a maximal analgesic effect.

Studies carried out using TRPM8-GFP mice have identified TRPM8 channel expression in distinct nerve terminal endings in the superficial layers of the epidermis (Dhaka et al., 2008). To our knowledge such anatomical characterisation has not been carried out for the 5-HT_{1B} receptor and it is not currently known if this receptor is expressed in peripheral nerve terminals. Further studies should also investigate the co-localisation of the TRPM8 channel and the 5-HT_{1B} receptor both in skin terminals and DRG, (a target attempted but not achieved here because of the inadequacy of currently available reagents). Since a 5-HT_{1B} receptor agonist exerted an analgesic effect when administered subcutaneously in a rodent model of inflammatory pain and icilin has been reported to be effective as an analgesic through topical administration (Granados-Soto et al., 2010; Proudfoot et al., 2006) peripheral routes conjoint of administration of TRPM8 channel and 5-HT_{1B} receptor agonists should be explored in a range of laboratory models of chronic pain to further validate the mechanism suggested by our results.

In conclusion, we have validated a novel QST technique for the measurement of mechanical nociceptive thresholds in laminitic horses, which holds potential for clinical assessment of the disease and for the assessment of analgesic efficacy. We have further shown that the pain state associated with equine laminitis might have a neuropathic component, which provides a basis for the incorporation of anti-neuropathic drugs in pain management protocols. We have also identified a novel modulatory mechanism of TRPM8 channel activity, which is mediated through 5-HT_{1B} receptors, which form a physical complex with these channels. This finding represents an important example of the newly developing concept of functional receptor/channel signalling complexes and potentially identifies a novel target for the development of analgesic treatments.

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APPENDIX: Publications arising from research

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Neuropathic changes in equine laminitis pain

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Abstract

Laminitis is a common debilitating disease in horses that involves painful disruption of the lamellar dermo-epidermal junction within the hoof. This condition is often refractory to conventional anti-inflammatory analgesia and results in unremitting pain, which in severe cases requires euthanasia. The mechanisms underlying pain in laminitis were investigated using quantification of behavioural pain indicators in conjunction with histological studies of peripheral nerves innervating the hoof. Laminitic horses displayed consistently altered or abnormal behaviours such as increased forelimb lifting and an increased proportion of time spent at the back of the box compared to normal horses. Electron micrographic analysis of the digital nerve of laminitic horses showed peripheral nerve morphology to be abnormal, as well as having reduced numbers of unmyelinated (43.2%) and myelinated fibers (34.6%) compared to normal horses. Sensory nerve cell bodies innervating the hoof, in cervical, C8 dorsal root ganglia (DRG), showed an upregulated expression of the neuronal injury marker, activating transcription factor-3 (ATF3) in both large NF-200-immunopositive neurons and small neurons that were either peripherin- or IB4-positive. A significantly increased expression of neuropeptide Y (NPY) was also observed in myelinated afferent neurons. These changes are similar to those reported in other neuropathic pain states and were not observed in the C4 DRG of laminitic horses, which is not associated with innervation of the forelimb. This study provides novel evidence for a neuropathic component to the chronic pain state associated with equine laminitis, indicating that anti-neuropathic analgesic treatment may well have a role in the management of this condition.

Keywords: Neuropathic pain; Dorsal root ganglion; Equine laminitis; Neuronal injury marker; ATF3

1. Introduction

Laminitis is a common cause of equine lameness involving one or more feet [11]. It is characterised by disruption of the dermo-epidermal laminar bond within the hoof (Fig. 1b and c) and subsequent structural weakness that can result in displacement of the pedal bone within the hoof capsule [41]. The pathogenesis of this disease is poorly understood but it is generally thought that vascular disturbances leading to ischemia-reperfusion injury of the lamellar structures are involved in the pathophysiology of laminitis [23]. Currently, no therapeutic regime is able to arrest or prevent its onset [42]. Moreover, laminitic pain can be difficult to control using traditional

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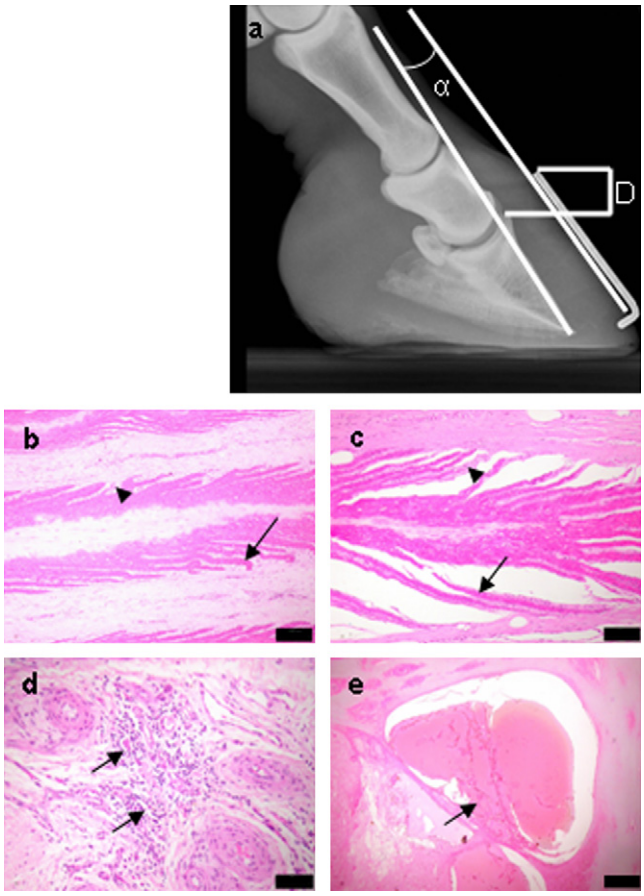

Fig. 1. (a) Latero-medial radiograph of laminitic equine digit showing rotation and vertical displacement (“sinking”) of the third phalanx relative to normal anatomy. Lines represent the standardised methods for measuring displacement (*D*) which is the distance (mm) between the proximal limit of the dorsal hoof wall and the extensor process of the distal phalanx, and the rotation angle (α) which is the angle between the dorsal surface of the distal phalanx and the dorsal surface of hoof wall [9]. (b) Haematoxylin/Eosin (H&E) stained histological section of the intact lamellar distal phalangeal apparatus in a normal horse showing the normal appearance of dermal (arrowhead) and epidermal (arrow) laminae 10×. (c) H&E stained histological section showing disruption and separation of the secondary epidermal (arrow) and secondary dermal (arrowhead) laminae in acute laminitis 10×. (d) Chronic laminitis. Mild inflammation in the laminar dermis, with small foci of lymphocytes in a perivascular location (arrows). H&E, original magnification 20×. (e) Chronic laminitis. Blood accumulation in the inner stratum medium. H&E, original magnification 4×. Scale bars (b–e) = 100 μm.

anti-inflammatory agents and euthanasia on welfare grounds is not uncommon [20,42,51]. Therefore, improved understanding of laminitis is much needed.

We hypothesized that the pathological inflammatory processes affecting the hoof laminae during laminitis also damage the sensory neurons innervating this region. Peripheral nerve injury can be associated with the generation of a neuropathic pain state characterised by allodynia (the perception of normally innocuous stimuli as painful), hyperalgesia (a heightened response to painful stimuli), spontaneous pain and a lack of response to conventional analgesics. A key factor in the neural plasticity underlying neuropathic (compared to inflammatory) pain is altered gene expression in sensory DRG neurons [10,21,58,59]. This can be demonstrated by an increase in expression of the neuronal injury marker ATF3, a member of the activating transcription factor/cAMP-responsive element binding protein (ATF/CREB) family, in sensory DRG cells [19,53]. Furthermore, phenotypic changes occur in primary afferent DRG neurons after peripheral nerve damage, resulting in altered expression of neuropeptides, including neuropeptide Y (NPY), the expression of which is induced from normally low levels in large diameter, neurofilament-200 (NF-200)-positive neurons following axotomy [21,54], nerve injury [31,36], demyelination [56] and streptozotocin-induced diabetes [46].

Injury to sensory nerves induces neurochemical, physiological and anatomical modifications to afferent and central neurons that are likely to contribute to chronic, sensitised neuropathic pain responses [58]. Such changes to the sensory neurons innervating the equine foot could lead to a clinically relevant component of chronic pain as it would explain the limited effectiveness of conventional analgesics in the treatment of laminitic pain [20].

Therefore, the aims of this study were to identify and quantify equine laminitic pain using objective behavioural assessment, characterise peripheral nerve damage in the lateral digital nerve and demonstrate potential nerve injury-associated alterations in protein expression in DRG sensory neurons innervating the feet of horses with laminitis.

2. Methods

2.1. Behavioural observations in laminitic and clinically normal horses

In order to define and quantify the behavioural characteristics of equine laminitis, we carried out continuous video monitoring over 3 days to compare behaviours in laminitic and normal horses.

Seven horses admitted for management of refractory laminitis were selected using the following clinical criteria: animals must have displayed clinical signs consistent with this disease including multi-limb lameness, increased amplitude of the digital pulses, warmth across the dorsal hoof wall and a laminitic gait [50]. For details of all laminitic horses used in this study see Table 1.

Latero-medial radiographs of the fore limb digits were obtained from each of these horses [7]. The position of the pedal bone within the hoof capsule was evaluated both subjectively and objectively by an experienced equine clinician using standard measures (Fig. 1a).

Informed client consent was obtained in writing prior to the onset of data collection. Laminitic horses received phenylbutazone (PBZ) twice daily at 08:00 and 20:00 h (Equipalazone Arnolds, UK; 2.2–4.0 mg kg^{-1}) and intramuscular

Table 1
Details of horses used in the study

Horse group/No.	Sex	Time from onset	Possible precipitating/concurrent conditions	Prior treatments
Laminitic 1	MN	1 month	Obese, increased liver enzymes	PBZ, ACP, NG, SS, RF, A, T
Laminitic 2	F	2 months	Obese	PBZ, ACP, RF, FT, SS
Laminitic 3	F	Recurrent >1 year	Obese	PBZ
Laminitic 4	F	2 days	None known	ACP, SS, F, A
Laminitic 5	MN	Recurrent >1 year	Grain overload	PBZ, F, SS, R, P
Laminitic 6	M	Recurrent >1 year	Obese	PBZ, RF, SS, NG, FT
Laminitic 7	MN	Recurrent >1 year	Access to rich pasture	PBZ, SS
Laminitic 8	MN	1 month	None known: prior history unknown	PBZ, FT, ACP, NG, SS, RF, A
Laminitic 9	MN	Recurrent >1 year	Euthanasia requested for chronic condition	PBZ, FT, RF
Laminitic 10	MN	Recurrent >1 year	None known: prior history unknown	PBZ, FT
Laminitic 11	MN	Recurrent >1 year	None known: prior history unknown	PBZ, RF
Laminitic 12	MN	Recurrent >1 year	None known: prior history unknown	PBZ, RF

Sex abbreviations: MN, male neutered; M, male intact; F, female.
Treatment abbreviations: PBZ, phenylbutazone; F, flunixin; A, aspirin; ACP, acepromazine; SS, solar supports; RF, remedial farriery; FT, foot trimming; NG, nitroglycerin (vasodilatory therapy); R, rehydration therapy; T, Trilostane (modifier of steroidogenesis); P, procaine penicillin + neomycin sulphate.
Estimated weight range for laminitic horses: 250–550 kg; age range: 6–21 years. Control horses used were 3 females, 3 neutered males and an intact male. Estimated weight range for control horses: 350–600 kg; age range: 8–19 years.

acepromazine three times daily at 08:00, 16:00 and 24:00 h (ACP Novartis, UK; 0.02–0.04 mg kg^{-1}).On the day of admission to hospital the timing of drug administration varied between individuals. Pedal bone support (Styrofoam Solar Support System™/Lilypads™) was provided at the clinician's discretion. Subjects participated in the study for a maximum of 3 days. Seven age, type and sex-matched horses, which were considered 'pain free' (control group), were stabled directly opposite the laminitic horses and recorded simultaneously in order to account for extraneous effects on behaviour. All horses were maintained on shavings and had free access to water. Laminitic animals were fed restricted rations of soaked hay, as is standard procedure, whereas control animals received haylage *ad libitum*.

Twenty-four-hour time-lapse video equipment (AG-6124, Panasonic) was used to record undisturbed behaviour in each stable. Point samples of 1 h duration were taken at 8-h intervals, at 06:00, 14:00 and 22:00 h, for 3 days starting at 14:00 h on Day 1. Samples were analysed continuously for duration of state and frequency of event behaviour (The Observer™ vs. 4.1, Noldus Information Technology, The Netherlands).

Two behaviours were selected for statistical analysis as being representative of the behaviours where changes were most likely to be observed [43,44]. Frequency of 'forelimb lifting' (as lifts min^{-1}) was defined as the raising and lowering of a forelimb, without locomotion and was adjusted for total time spent standing. 'Proportion of time spent at the back of the box' was defined as time spent positioned in the furthest 50% of the box, away from the entrance to the stable.

On a repeated dose regime, peak PBZ concentrations occur between 2 and 6 h following administration, although individual variation is high [17]. In the present study a 12-h dosing regime was used, minimising variation and increasing the probability of the maintenance of a 'steady state'. For sample point analysis of behaviours, data were collected at three different time points to reflect an expected minimum plasma PBZ concentration (06:00 h) and shortest (2 h post-administration – 22:00 h) and longest (6 h post-administration – 14:00 h) times for peak PBZ concentrations were chosen. In addition, to evaluate some of the possible effects of drug accumulation, analyses were repeated just using data from day 1, days 1 and 2 and days 1, 2 and 3.

2.2. Morphological investigations

Lateral digital nerves were obtained from five horses euthanised on clinical grounds due to laminitis which was either recurrent or refractory to therapy and also from four horses which had no history of forelimb lameness that were euthanised for clinical reasons other than forelimb pathology (control group). The lateral digital nerves were removed from the forelimb (3 cm long segments) at the level of the proximal sesamoid bone and fixed for 4 h in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, post-fixed in OsO_4, and embedded in Araldite. For light microscopy, 1 μm resin sections of the nerve were stained with Toluidine blue and three fascicles were chosen at random by bright-field microscopy. Ultra-thin (80 nm) sections were stained with uranyl acetate and lead citrate and examined on Phillips BioTwin electron microscope (FEI, UK Ltd, Cambridge, UK). Electron microscope (EM) images of cross-sections of fascicles within each nerve (areas ranging between 6732 and 47,215 μm^2) were analysed by eye by a trained, blinded observer using Image Tool 3.0 (UTHSCSA, USA). The total area of the nerve sections and the percentage of the nerve area occupied by nerve fascicles were calculated in order to investigate any differences between normal and laminitic digital nerves that might reflect oedema and therefore affect the quantification of axon density. The number and axon diameter of intact myelinated fibers was calculated as well as the percentage of damaged myelinated fibers, defined as those with a severe disruption of the myelin sheath and/or axonal degeneration. Myelin sheath thickness was measured and *G*-ratio of axons was calculated by dividing the axonal diameter by the total diameter of axon plus myelin sheath. The proportion of A-fibers with continuous Schwann cell cytoplasm (an abnormal morphological feature previously described by [8]) was also determined. C-fibers were identified as small-diameter unmyelinated fibers, surrounded by Schwann cell cytoplasm.

The total number of C-fibers was calculated as well as the percentage of solitary unmyelinated fibers and the number of unmyelinated fibers per Remak bundle. All analysis was carried out on identity-concealed samples.

2.3. Immunohistochemistry

DRG from cervical segments eight (forelimb innervation) and four (non-forelimb innervation) from the same horse were obtained post-mortem from the five laminitic horses and four control horses. The tissue was snap frozen and embedded in OCT embedding matrix (CellPath plc., Powys, Wales, UK). Cryostat sections of C8 DRGs (15 μm) were thaw-mounted on poly-L-lysine slides (Merck-BDH).

DRG sections were pre-incubated for 1 h at room temperature in 0.1 M PBS, pH 7.4, buffer containing 0.2% Triton X-100, 2% fish skin gelatin and 10% normal goat serum; and then incubated overnight at 4 °C with primary antibodies diluted in the same buffer. For co-localisation of the peptide NPY or ATF3 with the myelinated cell marker neurofilament 200 kDa (NF-200) [27,32], or either of the unmyelinated cell markers, peripherin or isolectin B4 (IB4) [18,33], antisera/lectin were used at the following concentrations: rabbit anti-NPY (1:250; Peninsula Laboratories Inc, Belmont, CA, USA); rabbit anti-ATF3 (1:300; Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse monoclonal anti-NF-200 (1:400; clone N52; Sigma); mouse monoclonal anti-peripherin (1:250; Chemicon International, Harlow, UK); IB4 from *Bandeiraea simplicifolia* (1:400; Sigma). Sections were then washed in buffer and incubated at room temperature for 2 h with Alexafluor 488-labelled goat anti-mouse IgG (1:500; Molecular Probes Europe BV, The Netherlands), Alexafluor 568-labelled goat anti-rabbit IgG (1:1000; Molecular Probes Europe BV, The Netherlands) or Alexa Fluor 488-labelled streptavidin (1:200). Three washes in 0.1M PBS were performed before the addition of To-Pro3 cyanine nucleic acid stain (Molecular Probes Europe BV, The Netherlands). Three final washes in 0.1 M PBS were conducted before cover-slipping with Vecta-Shield (Vector Laboratories, Burlingame, CA, USA). Control sections were processed as above omitting the primary reagents.

Observations were made and sections photographed on an Olympus microscope equipped for epifluorescence. All counts of profiles labelled for immunopositive cells were performed by the same observer (who was blinded to sample treatment) on randomly selected, 15 μm sections of DRG from each of the animals in each group. Every sixth section was selected to ensure that measurements were taken only once for each cell. Results were expressed as the proportion of labelled profiles per total number of single or double-labelled profiles from all sections, 95% confidence intervals (CI) are indicated.

2.4. Western blots

C4 and C8 DRG were taken from laminitic horses ($n = 3$). Whole lysate preparations were prepared by homogenising tissue in 20 volumes of Laemmli lysis buffer (Tris (tris-hydroxymethylaminoethane, 50 mM, pH 7.4), 5% mercaptoethanol and 2% sodium dodecyl sulphate (SDS)), boiled for 5 min and frozen. Western blotting was carried out as described previously [15]. Blots were incubated with rabbit polyclonal primary antibodies to ATF3 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and detected by peroxidase-linked secondary antibody and enhanced chemiluminescence. The ubiquitous housekeeping enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:750, Chemicon) was monitored as a control for protein level normalisation. Quantitative densitometry analysis of protein bands was performed using the ScanAnalysis (Elsevier) program.

2.5. Statistical analyses

Linear mixed-effect models were used to determine any differences between laminitic and control horses in the frequency of lifting the forelimb and time spent at the back of the box, in order to account for the repeated sampling of the same horses [40]. The ID of the horse that the samples came from was entered as a random effect. Laminitic/control, time point in experiment and time of day were entered as fixed effects. Prior to analysis of the forelimb lifting results, the data were square root-transformed to achieve normalisation of the residuals.

For the analysis of percentages of damaged A-fibers, A-fibers with Schwann cell cytoplasm and solitary unmyelinated fibers, only one meaned value per horse was obtained, and therefore repeated sampling has not taken place. Repeated measures of mean axon diameters in myelinated and unmyelinated fibers and thickness of myelin sheath were taken in both control and laminitic horses. Therefore, linear-mixed effect models were also used to determine any differences between (i) mean axon diameters in myelinated and unmyelinated fibers; (ii) thickness of myelin sheath from laminitic and control horses. Multiple measurements per horse were also taken of the number of fibers per Remak bundle but as the data were integers, differences in the number of fibers per Remak bundle were analysed using generalised linear mixed-effect models with Poisson errors. Only a single measurement per horse of the percentage of damaged A fibers; A fibers with continuous Schwann cell cytoplasm and solitary unmyelinated fibers was taken, therefore simple logistic regressions were employed to determine the differences between control and laminitic horses.

Differences in total nerve area occupied by fascicles between normal and laminitic horses were investigated using a Student's *t*-test, and differences in the percentage of nerve area occupied by fascicles between normal and laminitic horses by general linear models with binomial errors. Any differences in the proportion of labelled profiles were assessed by χ^2 analysis. Mann–Whitney non-parametric tests were used to analyse fiber density. Immunoblot data were analysed using a matched pair *t*-test. All analyses were carried out in S-PLUS 6.0 (Insightful, Seattle, USA) and SigmaStat 2.03 (SPSS Inc., USA). In all cases $p < 0.05$ was taken to indicate statistical significance, and degrees of freedom associated with any tests are denoted by subscripts.

3. Results

3.1. Radiographic abnormalities associated with laminitis were seen in all the laminitic horses

The horses clinically diagnosed with laminitis displayed radiographic evidence of this disease when the radiographs were evaluated objectively [7]. The angle

between the dorsal hoof wall and the dorsal surface of the distal phalanx was increased when compared to normal values (Fig. 1a). Means (±SD) values from the laminitic group were 8.2° ± 3.0° (normal values −0.86° ± 2.4°) [9]. Assessment of the *D* distance between the extensor process of the distal phalanx and the coronary band also showed a marked increase in the laminitic group ($D = 16.4 \pm 4.9$ mm) when compared to normal values (4.1 ± 2.17 mm) [9]. Histological sections of laminitic tissue also indicated inflammatory changes (Fig. 1d).

3.2. Laminitic horses display quantifiable abnormal behaviours

3.2.1. Data analysis: Forelimb lifting

When considering overall data, laminitic horses show a statistically significant increase in the mean square root frequency of forelimb lifting ($F_{1,12} = 11.5$, $p = 0.005$; Fig. 2a) adjusted for time spent standing compared to control horses recorded in the same environment over the same time period. Fluctuations in the frequency of this behaviour occurred in both groups over the period of observation, but the pattern of such changes did not differ significantly between control and laminitic horses ($F_{1,140} = 3.6$, $p = 0.059$).

3.2.2. Data analysis: Proportion of time spent at the back of the box

Laminitic horses spent significantly more time at the back of the box than control horses ($F_{1,12} = 6.1$, $p = 0.03$). There was no difference between the two groups in how behaviour altered throughout the study ($F_{1,148} = 0.2$, $p = 0.683$) (Fig. 2b). Time spent at the back of the box was markedly higher in laminitics than in controls at both 06:00 and 22:00 h but not at 14:00 h (days 2 and 3), when assessing individual sample point data. This effect is not seen at 14:00 h on day one, probably because drug administration regimes were not well-established at this time.

3.3. Distinct morphological abnormalities in both myelinated and unmyelinated peripheral nerve fibers innervating the hoof, in the lateral digital nerve of laminitic horses

The lateral digital nerves at the level of the proximal sesamoid bone were examined from both normal and laminitic horses. A mean of 11.65% (range 8.75–14.35) of the total fascicle area from each nerve section was analysed. EM analysis of three randomly selected fascicles per lateral digital nerve revealed morphological differences in both the myelinated and unmyelinated fiber populations in laminitic compared to non-laminitic horses (Table 2). Abnormalities in the shape of surviving axons and disruption of the myelin sheath, with accumulation of lipid droplets and myelin debris, were observed. The most obvious quantitative feature appeared to be a significant reduction in the number of both unmyelinated (−43.2%) and myelinated fibers (−34.6%) per unit area in laminitic compared to control horses ($p = 0.016$). In order to eliminate the possibility that any nerve oedema could artefactually lead to the appearance of reduced fiber density, morphometric analyses were carried out to measure the percentage area of nerve sections occupied by fascicles and total nerve area in normal compared to laminitic horses. No significant differences in the mean percentages were identified ($t_4 = -0.91$, $p = 0.414$) between normals 37% (95% CI: 35.7–38.1) and laminitics 41% (39.5–42.0). No significant differences in total nerve area were identified between normal and laminitic horses ($t_4 = -0.43$, $p = 0.692$). Further abnormalities were a significant decrease in the number of unmyelinated nerve fibers per Remak bundle ($F_{1,7} = 20.7$, $p = 0.003$) together with an increase in the percentage of solitary unmyelinated fibers in laminitics compared to normal horses ($\chi^2_1 = 35.7$, $p < 0.001$, Fig. 3 and Table 2b). The percentage of morphologically damaged myelinated fibers was significantly higher in laminitic horses when compared to normal horses ($\chi^2_1 = 31.5$, $p < 0.001$, Fig. 3 and Table 2a). Finally, the proportion of myelinated fibers with continuous Schwann cell cytoplasm was significantly higher in the laminitic horses ($\chi^2 = 338.4$, $p < 0.001$). No significant differences in myelin thickness or *G*-ratios were identified in laminitic compared to normal horses ($F_{1,7} < 0.5$, $p > 0.311$).

3.4. The neuronal injury marker ATF3 is selectively expressed in sensory neurons innervating the forelimb in laminitic horses

Using immunohistochemical analysis of the DRG cell population, we assessed the presence of ATF3 in comparison with the expression of NF-200 and either IB4 or peripherin. ATF3 expression was significantly increased in NF-200-positive C8 DRG cells from laminitic horses ($n = 3$), where 67% (15 sections, 304 cells, CI 58.8–69.9) of NF-200-positive DRG cells co-expressed ATF3, while only 10% (15 sections, 345 cells, CI 6.9–13.5) of NF-200-positive DRG cells in control horses ($n = 3$) co-localised ATF3 ($\chi^2_1 = 208$, $p < 0.001$) (Fig. 4). There was a significantly increased expression of ATF3 in IB4-positive C8 DRG cells in laminitic horses ($n = 3$), where 54% (five sections, 53 cells, CI 41.5–67.3) of IB4-positive C8 DRG cells co-localised ATF3 compared with 9% (five sections, 56 cells, CI 3.9–19.3) in control horses ($n = 3$) ($\chi^2_1 = 24.5$, $p < 0.001$). There was also a significantly increased proportion of peripherin-positive DRG cells that were positive for ATF3 in C8 DRG cells from laminitic horses, ($n = 3$), where 57% (nine

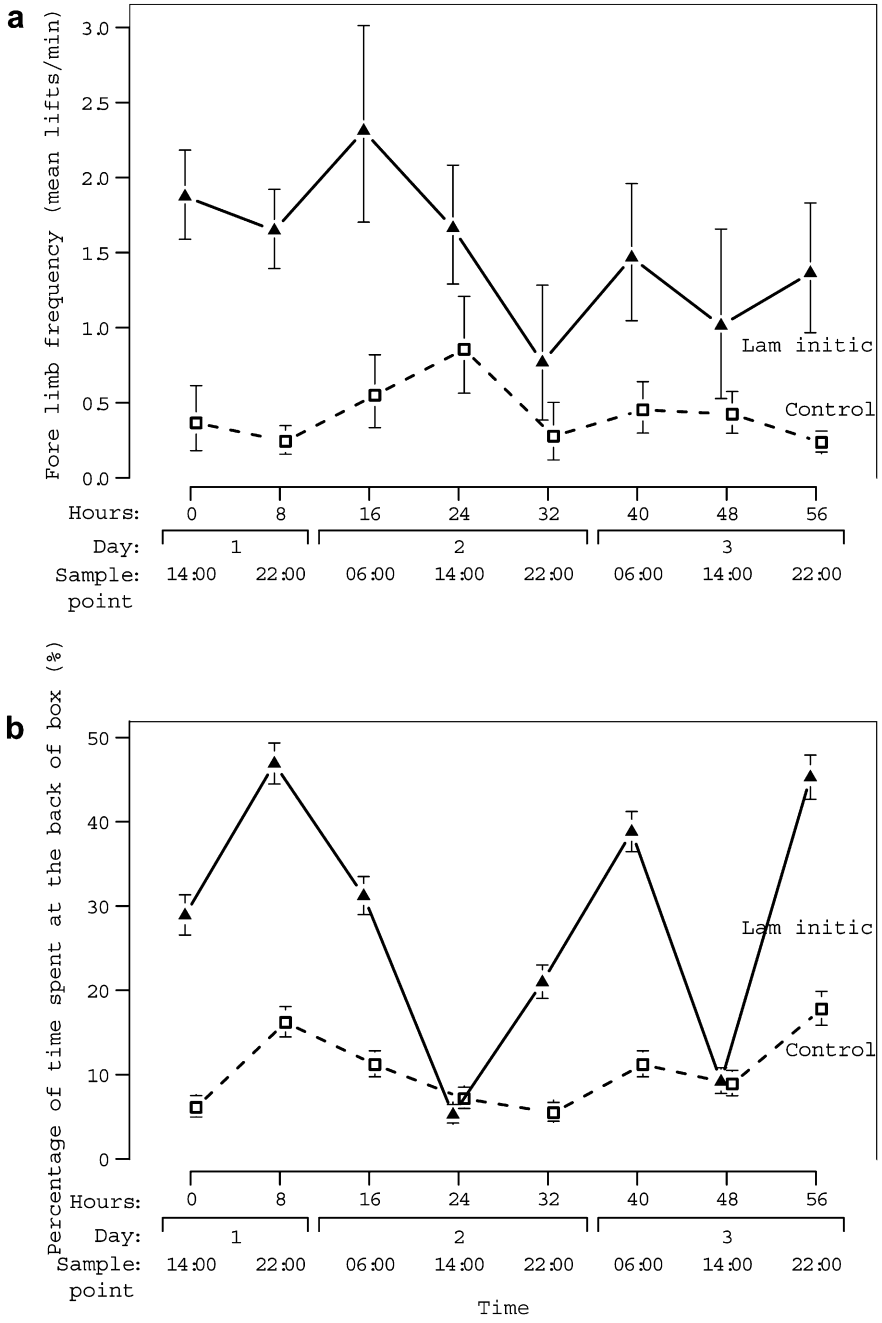

Fig. 2. Quantifiable pain behaviours in laminitic horses compared to control horses. Behavioural indices were recorded in laminitic horses (▲, solid line) ($n = 7$) and clinically normal (□, dashed line) horses ($n = 7$) over a period of 3 days, with 1-h observations at 06:00, 14:00 and 22:00 h. Phenylbutazone was administered each day at 08:00 and 20:00 h with supplementary acepromazine at 08:00, 16:00 and 24:00 h. (a) Forelimb lifting frequency adjusted for total time standing, expressed as lifts/min. When considering overall data, laminitic horses show a statistically significant increase in the mean square root frequency of forelimb lifting (±SE) adjusted for time spent standing compared to control horses recorded in the same environment over the same time period. (b) Proportion of time spent at the back of the box (away from the entrance), expressed as a percentage of time (±SE). Laminitic horses show a marked increase in the overall proportion of time spent at the back of the box, with marked differences from control horses at 06:00 and 22:00 h.

sections, 115 cells, CI 47.6–65.4) of peripherin-positive cells co-expressed ATF3 compared with 21% (nine sections, 127 cells, CI 13.8–29.4) showing double-labelling in control horses ($n = 3$) ($\chi^2_1 = 30.9$, $p = 0.001$; Fig. 4).

Accordingly, immunoblot analysis revealed a significant increase ($p < 0.05$) in ATF3 expression (expressed as mean percentage of GAPDH expression) in C8 DRG (38.9% (28.7–49.1)) in comparison to low levels in the control C4 DRG (4.6% (−0.5–

Table 2
Summary of myelinated and unmyelinated nerve fiber characteristics in normal and laminitic horses

Nerve fiber characteristics	Normal horses $n = 4$	Laminitic horses $n = 5$
(a) Myelinated fibers		
Mean number of fibers per 100 μm^2	0.52(±0.04)	0.34(±0.02)*
Mean percentage of damaged A fibers	16.40(±2.75)	30.08(±5.67)***
Mean percentage of A fibers with continuous (>40%) Schwann cell cytoplasm	17.45(±1.63)	72.46(±5.85)***
Mean axon diameter (μm)	5.38(±0.1)	5.08(±0.1)
Mean thickness of myelin sheath (μm)	1.06(±0.03)	1.09(±0.04)
(b) Unmyelinated fibers		
Mean number of fibers per l00 μm^2	5.77(±0.53)	3.28(±0.31)*
Mean number of fibers per Remak bundle	2.75(±0.07)	2.09(±0.03)**
Mean percentage of solitary unmyelinated fibers	30.14(±2.33)	38.06(±5.15)***
Mean axon diameter (μm)	1.36(±0.01)	1.28(±0.01)

Statistical significances are indicated by asterisks (*p value of <0.05, **$p < 0.01$, ***$p < 0.001$; linear mixed effects models, Mann–Whitney test – mean number of fibers per 100 μm^2). Values are expressed as means ± SEM.

9.7)) (Fig. 4b and f). The numbers of cells expressing NF-200, IB4 or peripherin were unaltered in laminitic DRG compared to normal horses (696 compared to 575 NF-200-IR cells, $n = 32$ sections, 115 compared to 127 peripherin-IR cells, $n =$ nine sections, 56 compared to 53 IB4-IR cells, $n =$ five sections, in normal compared to laminitic horses, respectively).

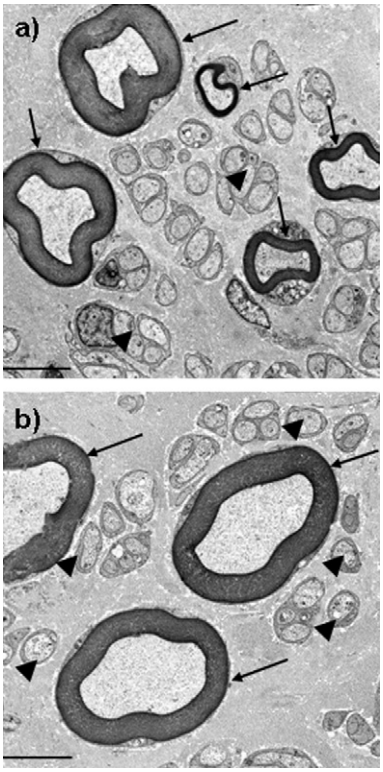

Fig. 3. Reduced myelinated and unmyelinated fiber density associated with laminitis. (a) Electron microscopy images of digital nerve from a normal horse. Arrows indicate intact, normal myelinated fibers. Arrowheads indicate clustered unmyelinated fibers in Remak bundles. (b) Electron microscopic images of digital nerve from a laminitic horse displaying reduced myelinated fiber density (arrows), lower numbers of C-fibers per Remak bundle, as well as increased numbers of solitary fibers (arrowheads) and increased collagen-filled space, compared to normal horse. Scale bars, 5 μm.

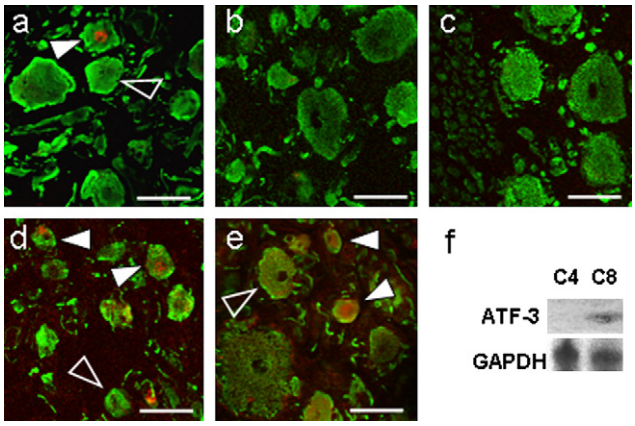

Fig. 4. (a–e) Immunohistochemical co-localisation of DRG neuronal subtype markers (NF-200 and peripherin, green) with neuronal injury marker, ATF3 or neuropeptide Y (NPY) (red) in C8 DRG (which receives forelimb innervation) of laminitic (a,d,e), or control horses (b) and co-localisation of NF-200 (green) with ATF3 (red) in C4 DRG (not associated with forelimb innervation) from the same horse (c). (a) In laminitic horses, there was an increased expression of ATF3 (red) in NF-200-positive DRG cells (green) compared to C8 DRG control (non-laminitic) horse (b) and C4 DRG from laminitic horse (c). Laminitic horses show expression of ATF-3 (red) in peripherin-positive (green) in DRG cells (d), while control horses do not (data not shown). Additionally, there was increased co-localisation of NPY (red) and NF-200 (green) in C8 DRG cells of laminitic horses (e) compared to control horses, where there was normally only sparse NPY expression (data not shown). Scale bars, 100 μm. White arrows show co-localised immunopositive cells. Open arrows show cell marker (NF-200 or peripherin)-positive cells lacking co-localisation. (f) Typical immunoblots of whole DRG lysates of laminitic horses ($n = 3$) show clear ATF3 expression in C8 but not C4 DRG. Levels of the housekeeping enzyme, GAPDH (lower blots), were unchanged.

3.5. Laminitis is associated with a distinctive pattern of expression of Neuropeptide Y (NPY) in sensory neurons

A significant increase in NPY immunoreactivity (NPY-IR) was observed in the C8 DRG of laminitic horses, where 77% (17 sections, 271 cells, CI 72.0–82.3) of NF-200-positive cells showed NPY-IR co-localisation, compared to only 10% (17 sections, 351 cells, CI 15.7–24.7) in control horses ($\chi^2_1 = 193$, $p < 0.001$; Fig. 4).

4. Discussion

Damage to sensory nerves has been linked to abnormal pain and heightened sensitivity to touch in a variety of clinical and experimental studies. In this study, we have quantified for the first time abnormal behaviours associated with equine laminitis which are indicative of a hypersensitive sensory state. Additionally, we provide novel evidence for changes associated with nerve damage in the sensory nerves innnervating the forelimb in laminitic horses, which are consistent with those reported in previously characterised neuropathic pain states.

4.1. The laminitic horses included in this study have digital pathology

Assessment of the radiographs from the laminitic horses identified pedal bone displacement (rotation or distal displacement) associated with laminar tearing. It was not possible to perform radiographic assessment of the control horses due to ethical and health and safety limitations, therefore data were compared to well-established normal data [9]. Chronic inflammatory changes were also observed (Fig. 1d) which have been previously shown to associate with sensory nerve losses in the skin [28,52].

4.2. Laminitic horses display chronically altered behaviour

We have quantified two behavioural changes associated with laminitis, which are suggestive of a chronic hypersensitive neuropathic pain state, characterised by the development of allodynia, hyperalgesia and spontaneous pain. Forelimb lifting represents an abnormal, *de novo* behaviour associated with laminitis, being at low levels or absent in the clinically normal horse. The overall scores for frequency of forelimb lifting were significantly greater in laminitic than control horses. Additionally, laminitic horses spent more time positioned towards the back of the box, a retiring behaviour that has also been associated with acute post-surgical limb pain [43]. This behaviour may represent a reluctance to engage in the external environment and preference to remain withdrawn from surroundings. The differences in retiring behaviour (percentage of time spent at the back of the box) showed the appearance of a marked cyclical pattern, although this was not seen with forelimb lifting, weight bearing when walking or general demeanour. The pattern apparent in time at the back of the box observations may correspond to NSAID dosing times, external environmental stimuli or possibly an intrinsic diurnal rhythm. This emphasises the need for behavioural testing at a number of regular intervals in order to correctly reveal specific changes. Moreover, the consistent deviations from normal behaviour over the three-day period confirmed that the NSAID analgesic regime was not consistently effective. When individual time points were evaluated, marked differences from control horses were consistently seen at 06:00 and 22:00 h observations.

4.3. Abnormal hoof sensory nerve morphology in laminitic horses is consistent with that reported in damaged peripheral nerves in neuropathic pain states

Two types of sensory receptors have been identified in the equine foot. Lamellated corpuscles, similar to Pacinian corpuscles, found primarily in the solar dermis of the heel, are low-threshold mechanoreceptors, which transmit their input via rapidly conducting, myelinated A-fibers [5]. Additionally, numerous naked nerve endings containing the neuropeptide, calcitonin gene related peptide (CGRP)-like immunoreactivity and other sensory neuropeptides such as substance P, neurokinin A and PHI (peptide histidine–isoleucine) were detected in the dermis of the dorsal hoof wall and sole [6]. Those containing CGRP are associated with nociception [48] and transmit via slowly conducting C-fibers. Axons from the hoof nociceptors and low-threshold mechanoreceptors as well as sympathetic fibers innervating the vasculature contribute to the sensory digital nerve.

Changes in any of these could potentially contribute to the etiology of the chronic laminitic pain state. To understand the mechanisms underlying laminitis pain and the incomplete response to anti-inflammatory analgesics, it is important to establish whether axonopathic changes may contribute. In laminitic horses, EM analysis identified marked decreases in myelinated and unmyelinated fiber numbers per unit area of digital nerve. This is unlikely to be due to nerve oedema as there were no significant differences between nerve section areas and fascicle areas in normal and laminitic horses. The marked increase in the number of solitary, unmyelinated fibers, may reflect demyelinated A-fibers or an absence of guiding pathways for regenerating C-fibers [4]. These morphological changes are consistent with those in laboratory neuropathic pain models, such as chronic constriction injury [2,14,16,34], crush injury [30], photochemically-induced ischemia [60] and diabetic neuropathy [12,25,29,49], thereby supporting our hypothe-

sis that peripheral nerve damage may contribute to laminitis pain.

Functional changes in the injured peripheral nerve have also been described in neuropathic pain models. The loss of large fibers in nerves from laminitic horses is important as part of the behavioural changes in neuropathic pain states may result from the loss of spinal inhibitory controls exerted indirectly by these afferents [2]. On the other hand, damage to both A and C-fibers appears to be necessary for the establishment of hyperalgesia and allodynia [14,60]. Electrophysiological studies further suggest that ectopic discharges in both spared C- and A-fibers may be important in maintaining neuropathic pain [1,14,24].

4.4. Sensory neurons of the forelimb in laminitic horses show characteristic changes associated with peripheral nerve injury

Following peripheral nerve damage, phenotypic changes occur in primary sensory neurons that may contribute to mediating central sensitisation [21,53]. We assessed whether key neurochemical changes in sensory neurons of laminitic horses are similar to those in rodent neuropathic pain models. The numbers of DRG cells expressing anatomical markers NF-200, peripherin or IB4 were unaltered. Following nerve crush injury, peripherin increases transiently in large DRG cells [57]. However, that model is associated with sensory loss [4] rather than the hypersensitivity seen here, as in other neuropathic and inflammatory pain states, where indeed peripherin expression is not upregulated [13,45,47].

Neuronal expression of ATF3, which is normally minimal, is upregulated after peripheral nerve injury and so acts as a marker of nerve injury [53]. The clear expression of ATF3 in NF-200, peripherin or IB4-positive sensory neurons of laminitic horses indicates neuronal damage to both A and C-fibers matching our observations of abnormal nerve morphology. These findings suggest that primary afferent injury associated with laminitis arises locally from the damage caused by hoof pathology, rather than from systemic disease, since ATF3 expression is low in neurons of unaffected limbs. Ischemia and ischemia/reperfusion are established causes of ATF-3 expression [19], so the ischemia-reperfusion injury of the digit thought to underlie acute laminitis [22] may also be involved in neuronal damage. We also found upregulated expression of NPY in large NF-200-positive DRG cells from laminitic horses, paralleling observations in other neuropathic pain models [26,31,36–38,46,55,56].

The novel findings reported here suggest that pathological changes occurring during laminitis bring about a chronic pain state with a neuropathic component. Although the mechanisms underlying the pathogenesis of laminitis remain to be fully elucidated, it is apparent that the early stages of laminitis are associated with vasoconstriction of the digital microvasculature [39] and inflammation [3]. Indeed, such pathological events can result in nerve damage (e.g. [35,60,61] and may thereby play a part in laminitis pain through the transition from acute inflammatory pain to a chronic syndrome with a neuropathic pain component.

Future studies will address the cellular and molecular mechanisms involved in the chronic laminitic pain state. These changes may be responsible, at least in part, for the limited efficacy of currently used anti-inflammatory therapy. The administration of anti-neuropathic agents may therefore achieve better pain management and improved quality of life in horses suffering from refractory laminitis.

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Quantitative assessment of increased sensitivity of chronic laminitic horses to hoof tester evoked pain

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Keywords: horse; equine pain; hoof tester; quantitative sensory testing; laminitis

Summary

Reasons for performing study: **To evaluate quantitative sensory testing (QST) of the feet of laminitic horses using a power-assisted hoof tester.**

Hypothesis: **Hoof Compression Thresholds (HCTs) can be measured reliably and are consistently lower in horses with chronic laminitis than in normal horses.**

Methods: **HCTs of chronic laminitic (n = 7) and normal horses (n = 7) were repeatedly measured using a hydraulically powered and feedback controlled hoof tester. Data from 2 tests, at 3 sites in both forefeet, during 3 sessions were collected and statistically analysed using linear mixed models.**

Results: **The mean ± s.e. HCT for the laminitic horses was 29.6 ± 3.5 kg/cm^2 and for horses in the normal group was 59.8 ± 4.3 kg/cm^2. Residual variance was the largest of the error components and was greater (P<0.001) for the normal horses; none of the other components significantly differed between the 2 groups. Averaging of HCTs from each foot could produce a test with intraclass correlation coefficients of 0.83 for the normal group and 0.87 for the laminitic group, with an estimated sensitivity of 0.94 and a specificity of 0.93. This test would permit detection with 80% power and 95% confidence of a reduction of over 40% in the difference in mean HCTs between laminitic and normal horses following effective treatment provided that the experimental groups are of 9 or more horses.**

Conclusions: **HCTs can be safely and reliably measured experimentally using this hoof tester. The level of variability found indicates that, under these conditions, treatments may need to produce at least a 40% improvement to be detected. Simplification of the hoof tester, training of the horse and repeated testing may permit the method to be used clinically to detect changes in the HCTs of individual laminitic horses but these potential improvements will require further investigation.**

Potential relevance: **Measurement of HCTs can provide an additional means for assessing the effectiveness of treatments for alleviation of chronic equine laminitis.**

Introduction

Accurate evaluation of pain in horses is of fundamental importance to equine welfare and for effective veterinary management of the equine patient. Clinical assessment of pain in the feet continues to be largely subjective and relies heavily upon the expertise and skills of the clinician. In attempts to improve assessment, quantitative sensory testing (QST) has been used in horses (e.g. Pippi and Lumb 1979; Kamerling *et al*. 1988; Chambers *et al*. 1990; Redua *et al*. 2002; Haussler and Erb 2006a,b; Haussler *et al*. 2007). QST methods involve the controlled application of standard noxious stimuli (mechanical or thermal) that evoke an easily recognised and consistent response, usually the start of a withdrawal response, which is used as the 'end-point' and defines the QST threshold. For a QST method to become established for clinical use it needs to be valid, sufficiently sensitive, reproducible and easily applied (Viñuela-Fernandez *et al*. 2007).

Equine laminitis is a common cause of lameness. The high prevalence and severe pain that can be caused by this condition means that laminitis is a serious welfare concern for the equine industry. Moreover, the pain experienced by horses suffering from laminitis can be chronically persistent and difficult to control using traditional analgesic/anti-inflammatory agents (Herthel and Hood 1999). Recent work suggests that the hypersensitive state associated with laminitis might be due not only to inflammation but also to damaged sensory nerves resulting in neuropathic pain (Jones *et al*. 2007).

Effective pain control is a prime and urgent target in treating laminitis and the development of new treatments relies on the ability to reliably and sensitively assess changes in the sensitivity of the feet to painful stimulation. Conventional, hand-operated, hoof testers are routinely used to assess the general sensitivity of the foot and to localise more sensitive sites within the foot. The application and interpretation of this test is heavily dependent upon the skill and experience of the operator. Attempts to improve the reliability of assessments in laminitic horses, by using a calibrated electronic hoof tester, have previously been made by Kamerling *et al*. (1988); in their studies, force was applied manually with potential for variability in its rate of application and in the maximum pressure applied by the hoof tester probe. Their

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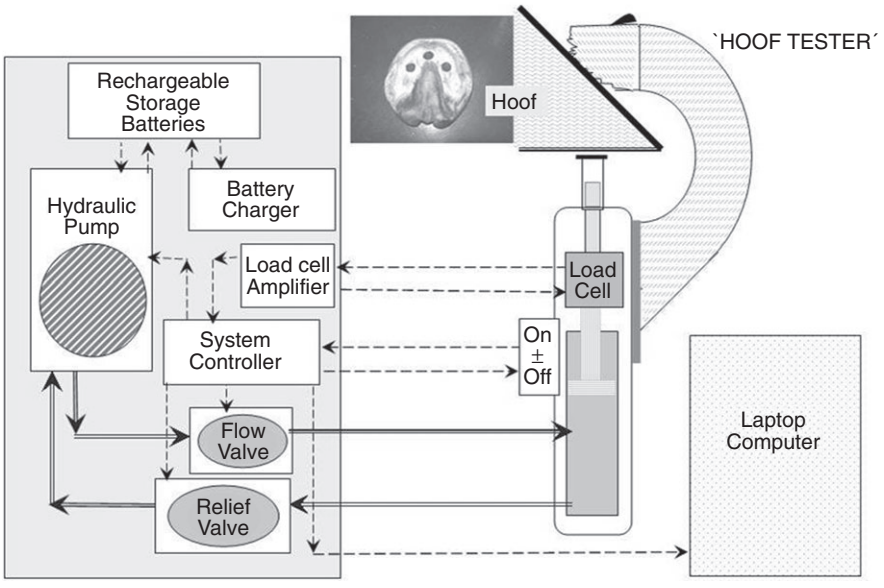

Fig 1: Hydraulically-powered and feedback controlled hoof tester: The diagram shows the hand piece, its connections to the control system and to a laptop computer. A hoof with surface marks on the sole is shown to indicate the location of the 3 sites (toe, medial and lateral) used for testing.

approach to quantitative hoof compression testing was used successfully to measure hoof compression thresholds at 21 different points across the sole in 6 horses with a variety of painful foot conditions including laminitis. This research group also used this quantitative approach to determine the effectiveness of analgesic treatments for chronic laminitis (Owens *et al*. 1995, 1996) but quantitative assessment has not yet become established for routine clinical assessment of laminitis.

This study aimed to: 1) evaluate the reliability and ease of use of a hydraulically-driven, feedback controlled, hoof tester for the quantification of mechanically evoked responses to noxious hoof stimulation in groups of normal and laminitic horses; and 2) record the range of thresholds and describe sources of their variation to help understand the difficulties involved in establishing quantitative hoof compression testing both for experimental and for routine clinical assessment.

Materials and methods

Animals

Seven horses suffering from refractory (chronic) laminitis for more than 2 months were selected, with their owners' consent, from patients of one of the authors (E.J.), at the Royal (Dick) School of Veterinary Studies and from horses under the care of the International League for the Protection of Horses (now World Horse Welfare), Belwade Farm, Aboyne, Aberdeenshire. As the laminitic cases were drawn from a clinical caseload it was not acceptable to stop therapy during the measurement period. Both forefeet were usually affected, although the results showed that the severity was generally not the same in each forefoot.

Seven normal (control) horses, each one matched as far as possible for size, age, breed and sex with one of the laminitic horses were selected, with their owners' consent, from amongst horses in local riding schools or belonging to members of staff of the Royal (Dick) School of Veterinary Studies.

Hoof tester

This instrument (see Fig 1) was designed and constructed by Robert Clark (Consultant Engineer, Roslin, Midlothian), and is powered by high-pressure fluid from a hydraulic pump through flexible hydraulic lines to a piston and a stimulating probe with a load cell in series. For stimulation, the flat surface (14.0 mm diameter) of the probe was applied to the sole of the hoof with the fixed, nonslip 'anvil' of the hoof tester applied opposite to it, on the more rigid dorsal wall of the hoof. A process controller (Eurotherm 3508, Eurotherm, UK) used the output of the load-cell to set a maximum (safety cut-off) and permitted the output of the load cell to be recorded to an Excel file (Microsoft Corporation, USA). The hoof tester was calibrated and adjusted to reliably produce a convenient and safe pressure vs. time output; an example of one test from one horse is shown in Figure 2.

Ethical considerations

It was agreed with the UK Home Office Inspectorate that any pain inflicted by these studies was of limited severity and of short duration, that the results were of potential benefit to the horses being tested and that the studies did not need to be licensed as scientific procedures under the Animals (SP) Act (1986). The limits to the intensity and duration of testing were based on those used by Kamerling *et al*. (1988) and on results from preliminary testing carried out with the approval of the ethical research committees of the Royal (Dick) School of Veterinary Studies and the University of Edinburgh.

Testing protocol

Testing was carried out in a quiet environment and the horses were allowed about 10 min to become accustomed to the environment. Horses were also habituated to the noise produced when switching the hoof tester on and off. None of the horses responded to this noise in a way that prevented testing.

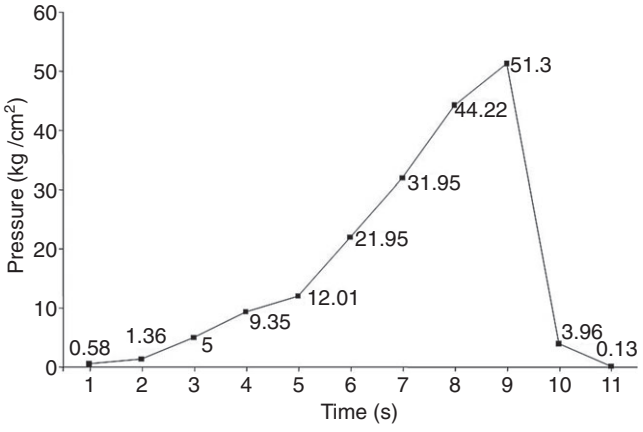

Fig 2: Hoof tester output: The graph shows the record of pressure vs. time for a single (typical) HCT; each change in force was logged from the load cell and data points for each second were averaged to produce the graph. The rate of increase in pressure for the last 4.0 s before reaching the HCT threshold was 9.8 kg/s/cm^2. The repeatability of this rate of increase is indicated by the standard deviation of the mean of 7 HCTs selected from 4 horses (10.8 ± 1.1 kg/s/cm^2).

The horses were tested while standing squarely on a firm level surface and were restrained, using a head collar, by an experienced horse handler. All testing was performed by the same veterinarian (I.V.-F.) after lifting the horse's forelimb and holding it as is standard practice in farriery. After cleaning the sole with a wire brush or hoof knife, the flat, circular, surface of the probe was applied to 3 sites, labelled 'toe' (half way between the apex of the frog and the white line), 'medial' (to the apex of the frog) and 'lateral' (to the apex of the frog) (Fig 1).

Application of gradually increasing pressure was started and stopped by a single 'on-off' thumb-switch on the handle of the instrument. The test threshold (HCT) was detected and pressure on the probe was immediately released by the operator at the onset of a withdrawal response of the limb. This response was generally detected by the operator as a sudden change in tone of the flexor muscles of the limb being held for testing. If the horse did not respond, pressure was automatically released through a relief valve, set by the process controller to open at 85 kg/cm^2 (maximum) to avoid lasting damage. The rate of application and the setting for the pressure relief valve (maximum) were chosen after preliminary studies (not reported here) in which the rate, duration and maximum pressure applied to normal horses were gradually increased. Pre- and post test lameness assessments, at a walk and trot, carried out by an experienced equine veterinary clinician (E.J.) did not detect any adverse effects.

The pressure exerted by the probe (kg/cm^2) was calculated from the force measured by the in-line, calibrated load cell and the contact area of the probe (1.54 cm^2). Pressure during each test was monitored graphically and recorded to an Excel file on a laptop computer.

Horses were tested in 3 test sessions separated by one week. Within each session, the forefeet of horses were tested twice at the 3 sites (toe, medial and lateral) with 10–15 s between tests and 2–3 min to change feet. Thus, 12 tests were obtained per horse on each session and a total of 36 tests/horse. Eight readings were not successfully recorded due either to operator error or failure to get the horse to cooperate. In total there were 250 measurements from the laminitic group and 246 from normal animals.

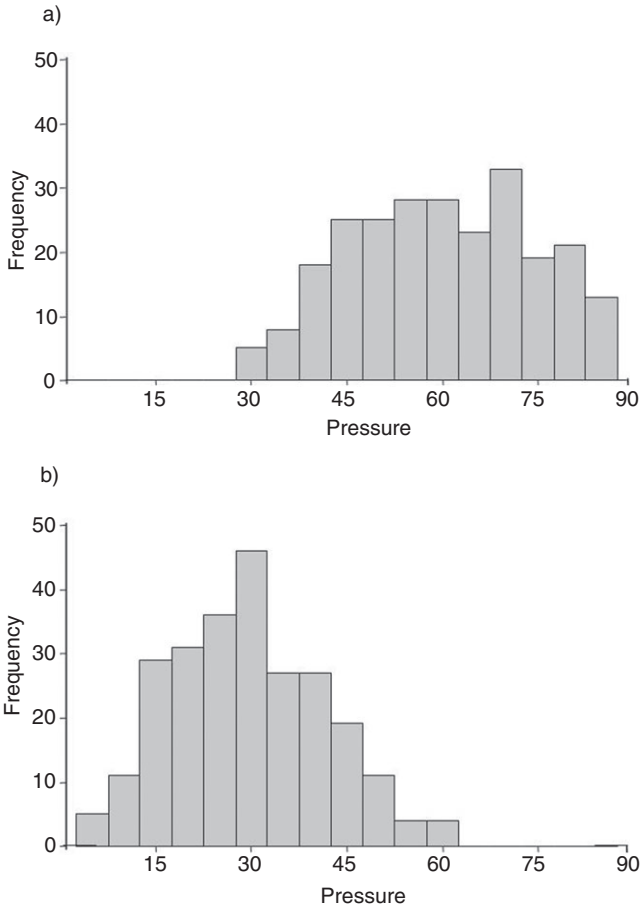

Fig 3: Histograms of the frequency of occurrence of hoof compression thresholds (pressure in kg/cm^2) obtained from testing of (a) normal (n = 7) and (b) laminitic (n = 7) horses.

Statistical analysis

Graphical analysis demonstrated that results could be treated as normally distributed in the statistical analyses (Fig 3).

Preliminary statistical analysis to investigate differences in hoof compression thresholds between normal and laminitic horses were carried out using a linear mixed model (Brown and Prescott 1999), treating Pressure as the response variable; Group (laminitic or control) and the other variates: Session (1, 2 or 3), Leg (left or right), Site (toe, medial, or lateral) and Test (1st or 2nd) and all interactions nested within Group, fitted as fixed effects and a factor encoding the sample matching (Matching), its interaction with Group (which is statistically equivalent to fitting an individual horse effect) and all possible interactions of the latter term with the other fixed effects, included as random effects. The fixed effects were progressively removed in a stepwise-type process until only statistically significant factors remained.

In order to appreciate the sources of variation better, data for the 2 groups were separately analysed using linear mixed models, which treated all relevant factors and their interactions as random effects. The estimated variance components are used to summarise the sources of variability in the data. The 16 estimated random effects can be found in the Table 2. Parsimonious models were also produced, by excluding components that were not associated with a statistically significant improvement in model fit, as quantified by the statistical deviance (Table 2). The values of the mean and

TABLE 1: Summary of measurements for normal and laminitic horses. The values are the mean hoof compression test thresholds ± s.e. for Group, Sites and Legs, obtained in 3 sessions with 2 tests at each site, expressed in kg/cm^2

Mean (± s.e.) kg/cm^2	Overall	Sites			Leg	
		Toe	Medial	Lateral	Left	Right
Normal	59.8 ± 4.3	60.7 ± 4.5	57.8 ± 4.5	61.0 ± 4.5	61.7 ± 4.4	57.9 ± 4.4
Laminitic	29.6 ± 3.5	28.2 ± 3.6	28.5 ± 3.6	32.0 ± 3.6	28.7 ± 3.8	30.5 ± 3.8

TABLE 2: Estimated variance components and s.e. for HCTs of normal and laminitic horses. Values in brackets were obtained after re-fitting of the REML model. Nonstatistically significant random effects are presented as either - or (-)

Random term	Normal horses		Laminitic horses	
	Component	s.e.	Component	s.e.
Animal	19.4 (19.1)	29.5 (29.6)	39.8 (37.8)	35.1 (35.0)
Session	0.0 (-)	- (-)	0.9 (-)	4.5 (-)
Leg	1.3 (-)	10.8 (-)	0.0 (-)	- (-)
Site	0.0 (-)	- (-)	3.0 (-)	4.6 (-)
Animal: Session	44.6 (38.7)	23.6 (23.4)	8.3 (10.5)	7.0 (6.9)
Animal : Leg	9.9 (10.3)	11.7 (13.6)	26.0 (26.1)	16.7 (16.4)
Session : Leg	9.1 (-)	11.2 (-)	1.7 (-)	3.3 (-)
Animal : Site	12.6 (12.9)	8.2 (8.2)	1.5 (6.5)	5.2 (5.0)
Session : Site	1.0 (-)	3.2 (-)	0.1 (-)	2.3 (-)
Leg : Site	0.0 (-)	- (-)	0.0 (-)	- (-)
Animal : Session : Leg	10.0 (22.0)	11.4 (15.1)	0.4 (0.5)	4.8 (5.4)
Animal : Session : Site	0.0 (-)	- (-)	4.5 (-)	6.3 (-)
Animal : Leg : Site	0.0 (-)	- (-)	2.7 (-)	5.3 (-)
Session : Leg : Site	0.0 (-)	- (-)	0.0 (-)	- (-)
Animal : Session : Leg : Site	0.0 (0.0)	- (-)	0.0 (5.1)	- (7.6)
Residual variance	102.6 (103.0)	10.6 (10.6)	65.1 (65.2)	7.5 (8.3)

variance components for each group of horses were used, in statistical models, to allow prediction of the benefits of reducing different sources of variability (errors) and prediction of the numbers of animals and tests that would be required to show treatment effects of various sizes.

To assess the repeatability of the method, intraclass correlation coefficients were determined by calculating the ratio of population variability to the total variability estimated from the final fitted model. All mixed modelling was carried out using Genstat 10th edition (VSNi, UK). Intraclass correlation coefficients, the sensitivity, specificity and area under the receiver-operating characteristic (ROC) curve were estimated parametrically using these variance components; calculations were carried out in Excel 2003.

Results

Differences between normal and laminitic horses

Histograms, presented in Figure 3, indicate that the threshold responses obtained from both normal (control) and laminitic horses can be described by a normal distribution.

A not unexpected and highly statistically significant ($P<0.001$) difference was found between the mean ± s.e. HCTs of normal (59.8 ± 4.3 kg/cm^2) and laminitic horses (29.6 ± 3.5 kg/cm^2) (Table 1).

None of the fixed effects were found to be statistically significant, except for the 3 way interaction Group.Site.Test ($P = 0.03$) where the effect was centred on a difference of less than 3 kg/cm^2 between the first and second HCTs of normal horses at the Lateral site.

Variance components analysis

Variance components, estimated separately for the HCTs of both normal and laminitic horses, are summarised in Table 2.

In both groups, variance components associated with Session, Leg and Site were consistently small. Subsequent refitting of the model without these terms showed that the properties of the model were not affected, showing that these models are consistent with the fixed effects model described earlier. However, these terms were statistically significant when they were estimated in interaction with the term 'animal', i.e. individual horses showed significant variability associated with these components.

For both normal and laminitic horses, the residual variance, estimated from the pairs of observations ('Tests' 1 and 2), was the largest of the variance components. This residual variance was significantly greater in the normal group than in the laminitic group ($P<0.01$ by z test). No statistically significant differences were found between normal and laminitic horses for the other variance components.

In normal horses the interaction providing the largest contribution to the total variance was that of 'animal by session', whereas for laminitic horses it was that of 'animal by leg' (Table 2).

Statistical test evaluation: variability between animals

Further statistical analysis was carried out by calculating the intraclass correlation coefficients (ICCs), i.e. the ratio of the population variability to the total variability in the data. The ICCs were 0.50 (normal group) and 0.57 (laminitic group). Therefore, for

TABLE 3: Power calculations: The table shows the numbers of repeated tests and horses in each group required to detect hypothetical improvements of 10, 20, 40 and 80% in the HCTs of laminitic horses towards normal values. A 100% improvement constitutes return to normality. Estimates were obtained from the results by modelling and a power of 80% with a confidence of 95% was assumed for the statistical testing. The table illustrates that, to detect an improvement of 40%, a group of at least 9 horses would be required and that each hoof would need to be tested at least 5 times before and after treatment

Percentage change, towards normal values, in the HCTs of a group of laminitic horses.	Number of tests				
	2	3	4	5	6
	Number of horses required				
10%	40	36	34	32	32
20%	21	19	18	17	16
40%	11	10	10	9	9
80%	6	6	6	5	5

both groups, around half of the observed variability was associated with interanimal variability.

The statistical sensitivity, specificity and area under the receiver-operating characteristic (ROC) curves were estimated as 0.89, 0.86 and 0.94, respectively.

The values of the mean and variance components for each group of horses were used to determine the benefits of averaging the pairs of tests (Tests 1 and 2); this showed that the ICCs were 0.67 (normal group) and 0.73 (laminitic group), with an estimated sensitivity of 0.91, a specificity of 0.89 and an area under the ROC curve of 0.96.

Statistical test evaluation: within animals and between sessions

To assess the repeatability of testing between sessions, ICCs were determined by estimating the ratio of variability associated with session to the total variability, while excluding variance components associated with interanimal variability. The estimated ICCs were 0.32 (normal group) and 0.14 (laminitic group). This indicates that, for both groups, when focusing on a single animal, little of the variability was between sessions.

Since most of the variability is between individual tests, total variability could be reduced by averaging the 2 HCTs for each site; the ICCs would then be 0.45 (normal group) and 0.20 (laminitic group).

The estimated variance components were also used to model a linear increase in the HCTs of laminitic horses over 3 sessions, until they become typical of those of normal animals, as would occur in clinical situations in response to effective treatment and healing. This hypothetical model was used to estimate the numbers of horses and/or test repeats (replicates) required to detect an increase (using a 2-sided *t* test) in the mean HCT of laminitic horses. The increase in HCTs between sessions was modelled and quantified as the percentage reduction in the initial difference between the mean HCTs of normal and laminitic horses; this difference disappears as laminitic horses reach normality. Assuming 80% power and 95% confidence for the statistical test, examples of the numbers of horses required are shown in Table 3 and it can be seen that if 5 HCTs were obtained from a laminitic foot (from a randomly selected site) in each session for a group of 9 horses, there would be an 80% chance of detecting a change in mean HCT of 40% towards the mean HCT of normal horses.

Discussion

This study confirms and extends measurements of thresholds, to noxious mechanical stimulation of the forefeet of lame horses, presented by Kamerling *et al*. (1988). They reported HCTs and the number of loci that were pain sensitive for 6 horses with lameness from various foot problems (they did not test normal horses). The range of HCTs found when converted to kg/cm^2 was from 18.9 $\pm$ 3.4 to 46.4 $\pm$ 12.0 with a mean $\pm$ s.e. of 24.9 $\pm$ 3.2 kg/cm^2, which is consistent with the mean reported here for laminitic horses (29.6 $\pm$ 3.5 kg/cm^2). The highest HCTs in their range are quite close to the mean reported here for normal horses (59.8 $\pm$ 4.3 kg/cm^2).

Kamerling *et al*. (1988) found decreases in HCTs (hyperalgesia) after an undefined number of repeated tests. Hyperalgesia was also found as a result of repeated hoof testing by Owens *et al*. (1995, 1996) when using the same calibrated hoof tester to measure the effectiveness of detomidine, ketoprofen and phenylbutazone as analgesics for chronic hoof pain. Owens *et al*. (1995, 1996) measured proportional changes in the HCTs of laminitic horses following treatment and expressed the effectiveness of treatments as the percentage increase in HCTs with reference to the baseline. They did not present absolute HCT values for either normal or laminitic horses.

Kamerling *et al*.'s (1988) calibrated hoof tester was applied manually over a period of about 2 s and the maximum pressure applied was often determined by the grip strength of the examiner. Results from the present study suggest that increasing probe pressure at the slower rate of about 10 kg/s/cm^2 (Fig 2) and prevention of overstimulation by automatic control of the maximum pressure, avoids excessive stimulation and prevents development of hyperalgesia. No damage was detected by clinical examination for lameness and no evidence was found, from decrease in the HCTs, that hyperalgesia developed as a result of repeated testing.

Hoof testing with this prototype instrument was only a little more difficult to achieve than with a standard manually-applied hoof tester. However, this hoof tester would be easier to use if the handpiece could be made free of connections by including both the power and recording systems without becoming more bulky.

The HCT thresholds for the group of chronically laminitic horses were generally <50% of those obtained for 'normal' control horses and this can be related to a widely accepted view of the severity of foot pain suffered by horses with chronic laminitis. Because of the severity of this pain, the study suggests that great care should be taken and independent ethical justification agreed if horses, found to have HCTs below 20 kg/cm^2, are to be re-tested.

In order for a new QST method to be established for routine assessment its reliability needs to be evaluated. This study approached this by analysing the sources of variation and calculating intracorrelation coefficients.

In both normal and laminitic horses, the 'residual variance' component, which reflects variability between Tests 1 and 2 in individual horses, was the largest element of the error and it was significantly greater in normal than laminitic horses (Tables 1 and 2). This finding may be attributed to the lower HCTs of laminitic horses and their increased reluctance to move voluntarily because of increased pain. The shorter time taken for each test in laminitic horses reduces opportunities for interfering variables to produce measurement errors and includes a reduced number of coincident voluntary movements of the horse, which obscure detection of the threshold. No published quantitative evidence has been found with

which to compare these differences in the variability of HCTs between normal and laminitic horses.

Variability between different horses was a large source of variability in the data, particularly for the group of laminitic horses. This is likely to be due to these horses being at different stages of progression of their laminitis and to differences in their responses to therapy.

In normal horses, one of the largest contributions to variability was a result of 'animal by session' interaction (i.e. the variability in HCTs of a horse, from one session to another). This was confirmed by calculation of intraclass correlation coefficients, which showed that, in normal horses, about one-third of the total variability is associated with between session variability. Such high variability in normal horses over time is a limiting factor in studies using HCTs for the measurement of treatment effects but suggestions as to how this may be overcome are included below.

The benefits of averaging of various sampling factors were modelled and averaging of HCTs from different sites in the same leg (foot) seems likely to be a useful strategy for improving the effectiveness of the test. The magnitude of the variance associated with an 'animal by leg' interaction in laminitic horses, might correspond to differences in the sensitivity of the left vs. right foot of individual animals. It is a common clinical finding that one of the feet is generally more severely affected than the other and this was also reported by Kamerling *et al*. (1988). Therefore averaging of HCTs from both forefeet together, would not be beneficial, for assessment of changes in the severity of laminitis.

In neither normal nor laminitic horses was there any strong evidence of differences in the statistical properties of HCTs measured at the 3 different sites. A single site, the toe (half-way between the apex of the frog and the white line), would be easier to use as a consistent anatomical location for testing and might provide less variable HCT measurements.

Differences between the first and second HCT in normal horses at the lateral site were statistically significant, although relatively small (<3 kg/cm^2) and it was not dependent on the right or left foot (leg). We believe this unexpected finding should be taken cautiously and it needs to be confirmed before further investigation of or speculation on its origin is pursued.

Owens *et al*. (1995, 1996) concluded that, because of underlying variability, relatively large and consistent changes in HCTs are required to show statistically significant improvements in response to treatment. The results presented here are consistent with this and provide further insight into the sources of the variability. No direct evidence was obtained here to support their proposal that quantitative measurement of HCTs provides a better means for detecting analgesic effects than subjective assessment of changes in lameness obtained using the Obel scoring method. More studies, in which both methods of assessment are used on the same horses at the same time, are required to test this proposal.

Results reported here indicate that when using HCTs for assessment of the efficacy of treatments for laminitis, measurement of normal horses should be included to help assess the completeness of their recovery.

In conclusion, this study shows that HCTs can be safely and reliably measured, under experimental conditions, using this hoof tester. The variability found in the HCTs indicates that treatments need to produce at least a 40% improvement in order to be detected in a group of 9 laminitic horses. Clinical application of the method, which requires measurement of meaningful changes in the HCTs of individual laminitic horses, may be achieved after successful investigation and implementation of one or more of the following potential improvements: 1) Decreasing the variability between measurements on the same foot of an individual horse by rigorous attention (including training) to: the horse's 'mental state', its behavioural responses to hoof testing, precise location of the instrument and improvements in the ability of the operator to reliably detect the HCT threshold (end-point) for a particular horse. 2) Finding well-defined and easily applied criteria for the rejection of HCT threshold measurements affected by coincident variables such as environmental disturbances or failure of the horse to cooperate. Criteria might include; rejection of all measurements at or exceeding the maximum (cut-off) pressure, and rejection of measurements greater or less than twice the s.d. of the 'running mean' HCT for the particular horse. 3) Accommodation of variability by averaging out some of the errors. Most variability occurs at the test stratum and we suggest that the averaging of 5 well-controlled HCTs, performed at one site (toe) on the sole of each foot, might be safely achieved without cumulative deleterious effects. 4) Because of the complexity and high cost of this hoof tester, development and testing of simpler and cheaper calibrated hoof testers, would help clinical exploitation of HCTs. We suggest that, in order to help ensure the welfare of horses being tested, specifications for and use of calibrated hoof testers should be consistent with the protocols and safe 'limits' described here. 5) After this study, to avoid the need to convert measurements to meet the kg/cm^2 standard, the contact area of the probe was reduced to 1.0 cm^2. The hoof tester, with this smaller probe, has been used on >20 laminitic horses without finding evidence of hyperalgesia (decreasing HCTs) or increases in the severity of lameness (clinical scoring) as a result of the testing.

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